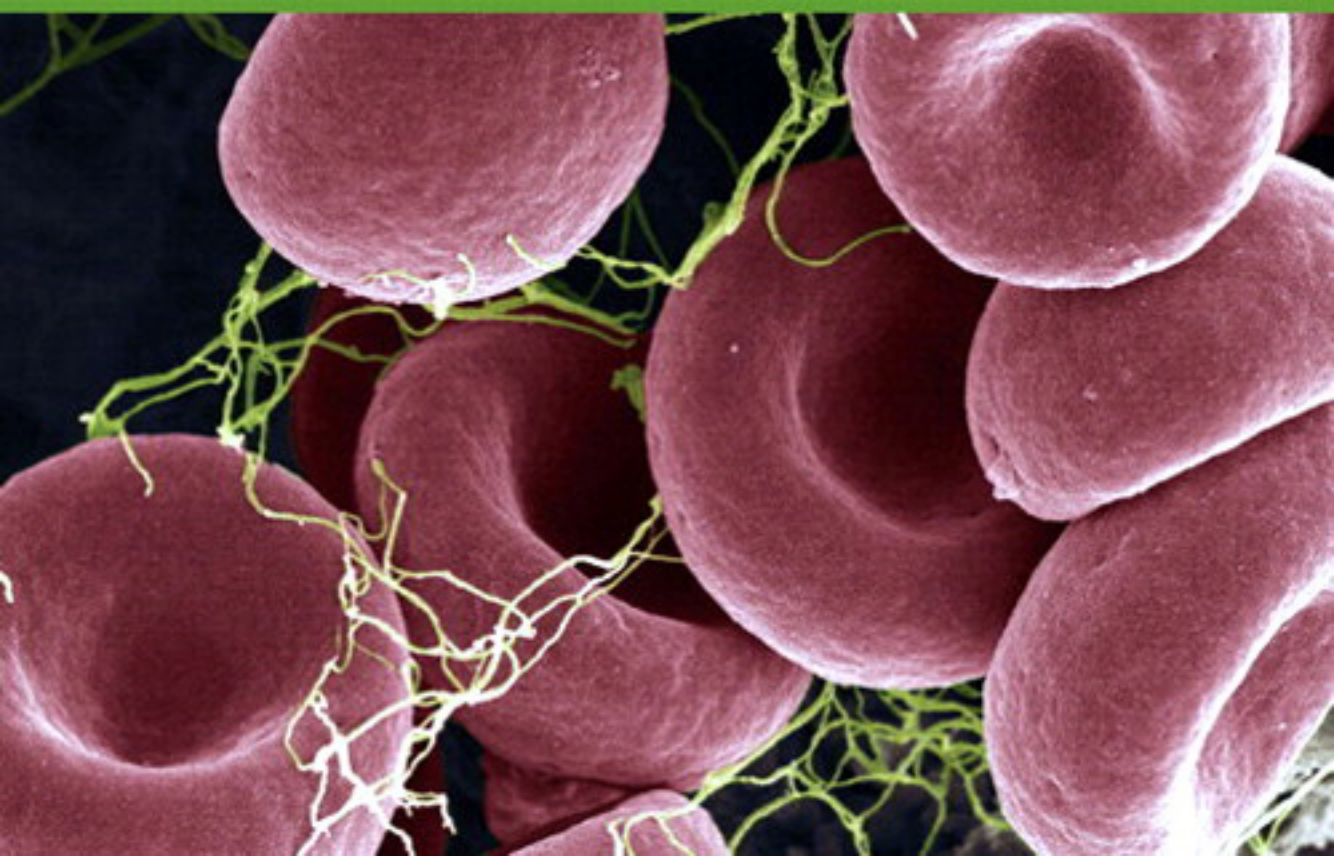
PEARSON CLINICAL LABORATORY SCIENCE SERIES

Clinical Laboratory Hematology

Third Edition

McKenzie | Williams

ELIZABETH A. GOCKEL-BLESSING, SERIES EDITOR



Data for reference values in these tables was compiled from multiple sources. These values will vary slightly among laboratories. Laboratories should derive reference intervals for their population and geographic location.

★ **TABLE A** Hematology Reference Values in Adults and Children (Hb, Hct, and RBC shown in conventional units; SI units in parentheses)

Age	Hb g/dL (g/L)	Hct % (L/L)	RBC × 10^6/mcL (μL) (×10^{12}/L)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Reticulocytes % (×10^9/L)
Adult							
Male	14–17.4 (140–174)	42–52 (0.42–0.52)	4.5–5.5 (4.5–5.5)	80–100	28–34	32–36	0.5–2.0 (25–75)
Female	12.0–16.0 (120–160)	36–46 (0.36–0.46)	4.0–5.0 (4.0–5.0)	80–100	28–34	32–36	0.5–2.0 (25–75)
Critical low limit	**6.6 g/dL, 1.7 SD**	**18%, 5 SD**					
Critical high limit	**19.9 g/dL, 2.7 SD**	**61%, 6 SD**					
Birth	135–200	0.42–0.60	3.9–5.9	98–123	31–37	30–36	1.7–7.0 (220–420)
2 weeks	130–200	0.39–0.65	3.6–5.9	88–123	30–37	28–35	1.0–3.0 (45–135)
							(same up to 1 year)
1 month	11–17 (110–170)	33–55 (0.33–0.55)	3.3–5.3	91–112	29–36	28–36	
2 months	9–13 (90–130)	28–42 (0.28–0.42)	3.1–4.3	84–106	27–34	28–35	
4 months	10–13 (100–130)	32–44 (0.32–0.44)	3.5–5.1	76–97	25–32	29–37	
6 months	11–14 (110–140)	31–41 (0.31–0.41)	3.9–5.5	68–85	24–30	33–37	
9 months	11–14 (110–140)	32–40 (0.32–0.40)	4.0–5.3	70–85	25–30	32–37	
1 year	13–14 (130–140)	33–41 (0.33–0.41)	4.1–5.3	71–84	24–30	32–37	
2–6 years	11.5–13.5 (115–135)	34–41 (0.34–0.41)	3.9–5.3	75–87	24–30	31–37	
6–12 years	11.5–15.5 (115–155)	35–45 (0.35–0.45)	4.0–5.2	77–95	25–33	31–37	

Reference intervals derived from combined data. Critical limits are the low and high boundaries of life-threatening values. Results that fall below the low critical limit and above the high critical limit are "panic values" or critical results that require emergency notification of physicians. These limits were derived by Dr. George Kost from a national survey of 92 institutions.

Source: Data from article by Kost GJ: Critical limits for urgent clinician notification at US Medical Centers. *JAMA*. 1990; 263:704.

★ TABLE B Age and Race-Specific Reference Intervals for Leukocyte Count and Differential[a]

	Birth	6 Months	4 Years	Adult	Adult of African Descent
Total leukocyte count ($\times 10^9$/L)	9.0–30.0	6.0–18.0	4.5–13.5	4.5–11.0	3.0–9.0
Segmented neutrophil: percent (%)	50–60	25–35	35–45	40–80	45–55
Absolute ($\times 10^9$/L)	4.5–18.0	1.5–6.3	1.5–8.5	1.8–7.0	1.5–5.0
Band neutrophil percent (%)	5–14	0–5	0–5	0–5	0–5
Absolute ($\times 10^9$/L)	0.5–4.2	0–1.0	0–0.7	0–0.7	0–0.7
Lymphocyte percent (%)	25–35	55–65	50–65	25–35	35–45
Absolute ($\times 10^9$/L)	2.0–11.0	4.0–13.5	2.0–8.8	1.0–4.8	1.0–4.8
Monocyte percent (%)	2–10	2–10	2–10	2–10	2–10
Absolute ($\times 10^9$/L)	0.2–3.0	0.1–2.0	0.1–1.4	0.1–0.8	0.1–0.8
Eosinophil percent (%)	0–5	0–5	0–5	0–5	0–5
Absolute ($\times 10^9$/L)	0–1.5	0–0.9	0–0.7	0–0.4	0–0.4
Basophil percent (%)	0–1	0–1	0–1	0–1	0–1
Absolute ($\times 10^9$/L)	0–0.6	0–0.4	0–0.3	0–0.2	0–0.2

[a]Compiled from multiple sources. Values may vary among sources and laboratories.

★ TABLE C Other Hematology Reference Values

Analyte		Reference Value
Immature reticulocyte fraction (IRF)		0.09–0.31
RDW		12–14.6
Platelet count		150–400 $\times$ 10^9/L
MPV		6.8–10.2 fL
Sedimentation rate		
Male	<50 years	0–15 mm/hr
	>50 years	0–20 mm/hr
Female	<50 years	0–20 mm/hr
	>50 years	0–30 mm/hr
Zeta sedimentation rate		
Male		40–52
Female		40–52
Cerebrospinal fluid		
Erythrocytes		0
Leukocytes		<5/mcL

Clinical Laboratory Hematology

Third Edition

Shirlyn B. McKenzie, PhD, MLS(ASCP)^{CM}, SH(ASCP)^{CM}

Department of Clinical Laboratory Sciences

University of Texas Health Science Center at San Antonio

J. Lynne Williams, PhD, MT(ASCP)

Biomedical Diagnostic and Therapeutic Sciences Program
School of Health Sciences

Oakland University

Consulting Editor:

Kristin Landis-Piwowar, PhD, MLS(ASCP)^{CM}

Biomedical Diagnostic and Therapeutic Sciences Program
School of Health Sciences

Oakland University

PEARSON

Boston Columbus Indianapolis New York San Francisco Upper Saddle River
Amsterdam Cape Town Dubai London Madrid Milan Munich Paris Montreal Toronto
Delhi Mexico City Sao Paulo Sydney Hong Kong Seoul Singapore Taipei Tokyo

Publisher: Julie Levin Alexander
Publisher's Assistant: Regina Bruno
Acquisition Editor: John Goucher
Development Editor: Andrea Klingler
Program Manager: Jonathan Cheung
Director of Marketing: David Gesell
Executive Marketing Manager: Brittany Hammond
Marketing Specialist: Alicia Wozniak
Project Management Lead: Cynthia Zonneveld
Project Manager: Patricia Gutierrez
Production Editor: Rebecca Lazure, Laserwords Private Limited
Senior Media Editor: Amy Peltier
Media Project Manager: Lorena Cerisano
Manufacturing Manager: Vincent Scelta
Manufacturing Buyer: Mary Ann Gloriande
Art Director: Mary Siener
Cover Designer: Carly Schnur
Cover Art: Science Photo Library—STEVE GSCHMEISSNER
Composition: Laserwords Private Limited
Printing and Binding: Courier/Kendallville
Cover Printer: Phoenix Color/Hagerstown

Library of Congress Cataloging-in-Publication Data
McKenzie, Shirlyn B., author.
Clinical laboratory hematology / Shirlyn B. McKenzie, J. Lynne Williams, Kristin Landis-Piwowar. — Third edition.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-0-13-307601-1 — ISBN 0-13-307601-6
I. Williams, Joanne Lynne, 1949- author. II. Landis-Piwowar, Kristin, author. III. Title.
[DNLM: 1. Clinical Laboratory Techniques. 2. Hematology—methods. 3. Hematologic Diseases—diagnosis. 4. Hematopoietic System—physiology. WH 25]
RB45
616.1'5075—dc23

2014015846

www.pearsonhighered.com

10 9 8 7 6 5 4 3 2 1

PEARSON

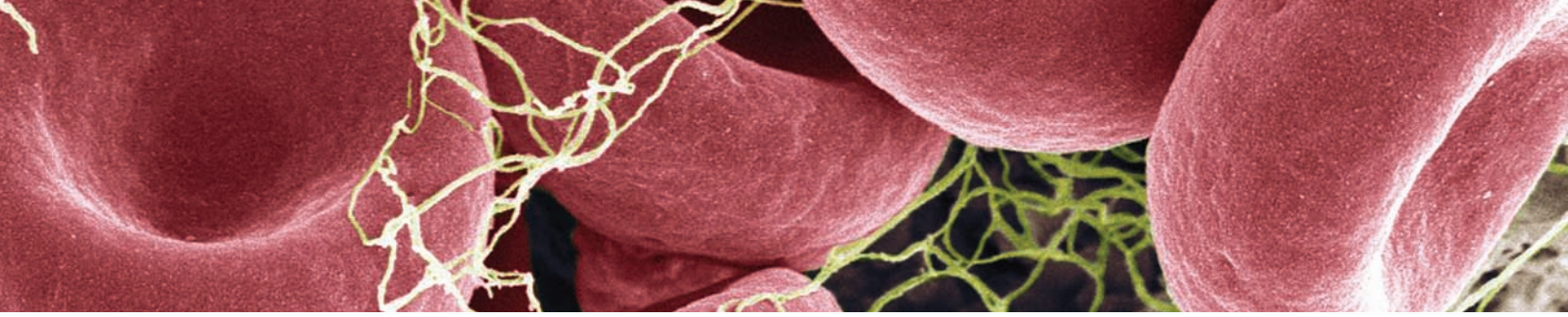
ISBN-10: 0-13-307601-6
ISBN-13: 978-0-13-307601-1

To my family, the wind beneath my wings, Gary, Scott, Shawn, Belynda, and Dora; my special grandchildren Lauren, Kristen, Weston, Waylon, and Wyatt; to the memory of my parents, George and Helen Olson.

Shirlyn B. McKenzie

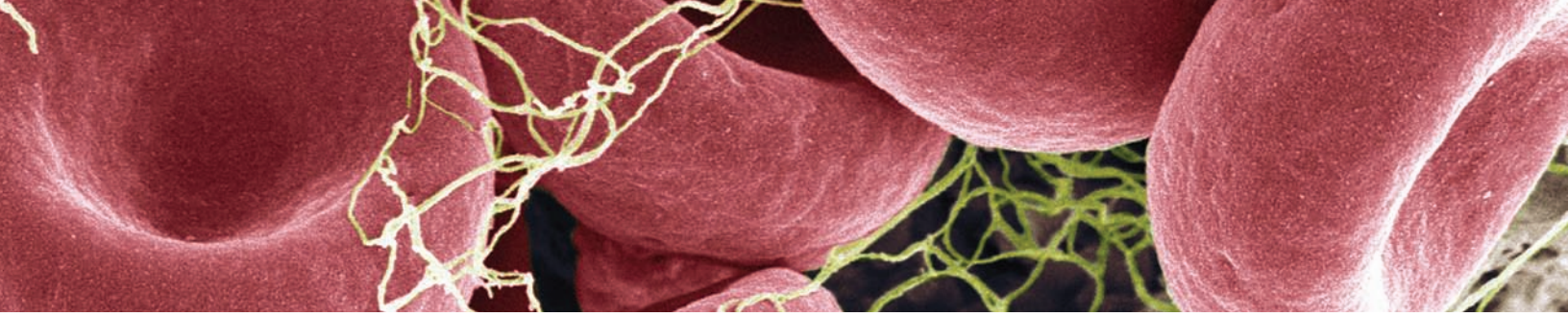
For my mother, Mary Williams, who gave her children roots as well as wings; for Lee, Laurie, Roger, and Richard, who sustain my roots; for Dulaney, Corie, Chris, Ava, and Holden, whom I love as my own; and to the memory of my father, David Williams.

J. Lynne Williams



Contents

APPENDICES

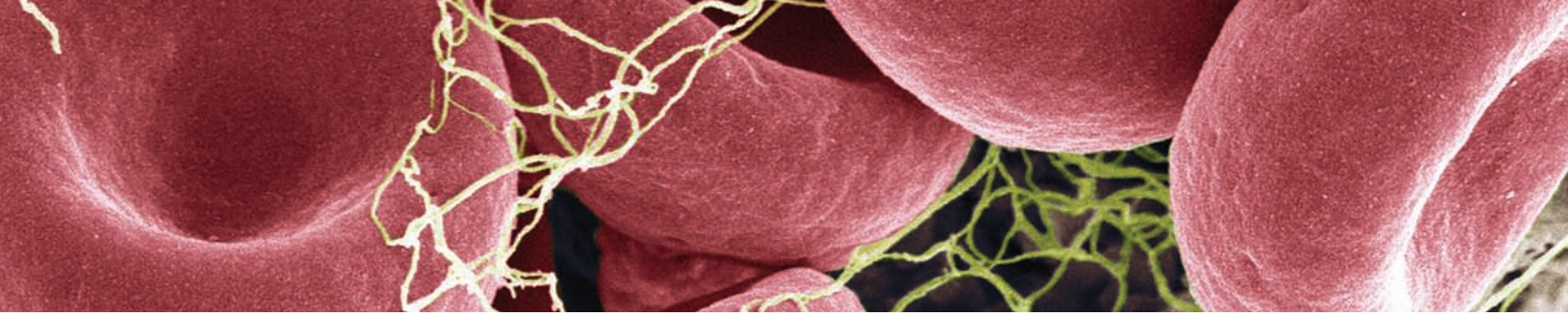


Foreword

Clinical Laboratory Hematology is part of Pearson's Clinical Laboratory Science (CLS) series of textbooks, which is designed to balance theory and practical applications in a way that is engaging and useful to students. The authors of and contributors to *Clinical Laboratory Hematology* present highly detailed technical information and real-life case studies that will help learners envision themselves as members of the health care team, providing the laboratory services specific to hematology that assist in patient care. The mixture of theoretical and practical information relating to hematology provided in this text allows learners to analyze and synthesize this information and, ultimately, to answer questions and solve problems and cases. Additional applications and instructional resources are available at www.pearsonhighered.com/healthprofesionsresources.

We hope that this book, as well as the entire series, proves to be a valuable educational resource.

Elizabeth A. Gockel-Blessing, PhD, MT(ASCP), CLS(NCA)
Clinical Laboratory Science Series Editor
Pearson Health Science
Vice Chair & Associate Professor
Department of Clinical Laboratory Science
Doisy College of Health Sciences
Saint Louis University



Preface

As with the first two editions, the third edition of *Clinical Laboratory Hematology* is designed to be a comprehensive resource that medical laboratory technician (MLT) and medical laboratory science (MLS) students can use in all their hematology courses. Laboratory practitioners will find the book a welcome resource to help them keep up with advances in the field. The book also is suited for use by students in other health care professions including pathology, medicine, physician assistant, and nursing. This edition is thoroughly updated to include the latest in advances in laboratory medicine. Each chapter has a similar format; the striking visual design makes it easy for readers to find information on each topic. Multiple supplemental learning tools for students and teaching resources for the instructor, including a website with resources available by chapter, are available. In summary, the book is not just a book but a package of learning tools.

ORGANIZATION

We believe that students must have a thorough knowledge of normal hematopoiesis and cell processes to understand the pathophysiology of hematologic/hemostatic diseases, evaluate and correlate laboratory test results, and ensure the appropriate utilization of the laboratory in diagnosis and patient follow-up. Thus, this book is organized so that the first 10 chapters give the students a comprehensive base of knowledge about blood cell proliferation, maturation, and differentiation and the processes that control hematopoiesis. Section One (Chapters 1–2) includes an introduction to hematology and hematopoiesis, including cell morphology and the cell cycle and its regulation. This introduction includes a description of cellular processes at the molecular level, which could be new material for some students and a basic review for others. The reader might want to review these chapters before beginning a study of neoplastic disorders. Section Two (Chapters 3–10) includes chapters on normal hematopoiesis, including a description of the structure and function of hematopoietic tissue and organs, erythropoiesis, leukopoiesis, and hemoglobin. In this third edition, the chapter on leukocytes is divided into two separate chapters: granulocytes/monocytes (Chapter 7) and lymphocytes (Chapter 8). An introductory chapter on platelets (Chapter 9) was added to this section to complete the discussion of normal blood cells. Details of platelet function and physiology are found in Section Eight, "Hemostasis." Chapter 10, "The Complete Blood Count and Peripheral Blood Smear Examination" is a new chapter that describes the information that can be gained about blood cells from these frequently ordered laboratory tests. Most of the remaining chapters refer to the tests that are described in this chapter.

The next three sections include discussions of hematologic disorders. Section Three (Chapters 11–20) begins with an introduction to anemia (Chapter 11). In this edition, we combined the introduction to anemia and the introduction to hemolytic anemia into one chapter because many anemias have a hemolytic component. This chapter is followed by chapters on the various anemias. Each anemia is discussed in the following manner: introduction, etiology, pathophysiology, clinical findings, laboratory findings, and therapy. This format helps readers understand what laboratory tests can help in diagnosis and how to interpret the results of these tests. Section Four (Chapters 21 and 22) covers the nonmalignant disorders of leukocytes. Section Five (Chapters 23–29) is a study of hematopoietic neoplasms. This section begins with an overview of these disorders to help students understand the classification, terminology, and pathophysiology of neoplasms and the laboratory's role in diagnosis and therapy. As a part of this section, we included a chapter on stem cell therapy (Chapter 29) because it is a frequently used therapy for these neoplasms and the laboratory plays a critical role in harvesting the stem cells and preparing them for transplant. Molecular studies are becoming a major diagnostic tool for neoplastic disorders and are discussed within each chapter as well as in the chapter devoted to molecular diagnostics (Chapter 42). Some instructors might prefer to cover Section Eight, the study of bone marrow (Chapter 38), flow cytometry (Chapter 40), cytogenetics (Chapter 41), and molecular diagnostics (Chapter 42) before teaching Section Five or integrate this material with Section Five. Some hematology courses do not include these topics, or instructors might not want to cover them in the depth presented in this book.

Section Six (Chapter 30) is a study of body fluids from a hematologic perspective and thus includes a large number of photographs of cells found in body fluids. This chapter has been reorganized and revised extensively to give a more complete perspective on body fluid analysis. Discussions of semen analysis and amniotic fluid lamellar body counts have been added. Additional photographs have been added to the online resources. Not all hematology courses include

this topic, but the chapter is written in such a way that it can be used separately in a body fluid course.

Section Seven (Chapters 31–36) is a study of hemostasis. Chapters on normal hemostasis include primary and secondary hemostasis and fibrinolysis. They are followed by three chapters on disorders of hemostasis. Chapter 36 describes the testing procedures for hemostasis, including information on automation. This chapter has been revised by laboratory coagulation specialists and describes an extensive collection of coagulation procedures; additional detailed information on hemostasis testing is available on the chapter's website. These procedures can be downloaded and used as is or adapted for use in student laboratories.

Section Eight (Chapters 37–42) includes chapters on test procedures that help in the diagnosis of hematologic disorders. Automation in hematology is included in Chapter 39. Extensive additional information is included on the book's website and includes step-by-step procedures for some tests, graphs, tables, figures, and printouts of abnormal results using various hematology analyzers. Chapter 42 is designed to introduce molecular procedures and their use in detecting various hematologic and hemostatic disorders. A background in genetics is suggested before students begin this chapter.

Section Nine (Chapter 43) is a thorough discussion of quality assessment in the hematology laboratory. Problems discussed include common abnormal results, errors, and alert flags. Corrective action to take to resolve these problems is described. Several excellent tables help to quickly find needed information. We suggest that these tables be read early in the course of study because they can be used periodically when attempting to interpret and correlate laboratory test results. Chapter 10 refers the reader to these tables because it discusses interpretation of test results and abnormalities in the CBC.

The text emphasizes the effective, efficient, and ethical use of laboratory tests. The clinical laboratory professional is in an ideal position to assist physicians in interpreting laboratory test results and choosing the best reflex tests to arrive at a diagnosis or evaluate therapy. Many laboratories develop algorithms to assist in these tasks. This text includes several algorithms that some laboratories use. To save page space in the text, some algorithms are on the website.

SUITABLE FOR ALL LEVELS OF LEARNING

The book is designed for both MLT and MLS students. Using only one textbook for both levels is beneficial and economic for laboratory science programs that offer both levels of instruction. It also is helpful for programs that have developed articulated MLT to MLS curricula. The MLS program can be confident of the MLT's knowledge in hematology without doing a time-consuming analysis of the MLT course.

Objectives are divided into two levels: Level I (basic) and Level II (advanced). MLT instructors who reviewed the objectives for this text generally agreed that most Level I objectives are appropriate for the MLT body of knowledge. They also indicated that some Level II objectives are appropriate for MLTs. MLS students should be able to meet both Level I and Level II objectives in most cases. If the MLS program has two levels of hematology courses—Level I and Level II—this book can be used for both.

All instructors, regardless of discipline or level, need to communicate to their students what is expected of them. They might want their students to find the information in the text that allows them to satisfy selected objectives, or they might assign particular sections to read. If not assigned specific sections to read, the MLT students may read more than expected, which is not a bad thing! The two levels of review questions at the end of each chapter are matched to the two levels of objectives.

The Case Study questions and the Checkpoints are not delineated by level. All students should try to answer as many of them as possible to assess their understanding of the material.

We recognize that there are many approaches to organizing a hematology course and that not all instructors teach in the same topic sequence or at the same depth. Thus, we encourage instructors to use the book by selecting appropriate chapters and objectives for their students based on their course goals. Each program should assess what content fits its particular curriculum. The layout of the book is such that instructors can select the sequence of chapters in an order that fits their course design, which might not necessarily be the sequence in the book. However, we recommend that the course begin with Sections One and Two and that the chapters "Introduction to Anemia" and "Introduction to Hematopoietic Neoplasms" be studied before the individual chapters that follow on these topics. The Background Basics sections help the instructor determine which concepts students should master before beginning each chapter. This feature helps instructors customize their courses. Some hematology courses might not include some chapters on subjects such as molecular techniques, cytogenetics, flow cytometry, and body fluids but they might be helpful in other courses.

As a note, this text uses mc as an abbreviation for micro, which replaces μ. Thus abbreviations of mcg, mcL, mcM replace those that use the Greek letter "mu" (μg, μL, μM).

UNIQUE PEDAGOGICAL FEATURES

The text has a number of unique pedagogical features to help the students assimilate, organize, and understand the information. Each chapter begins with a group of components intended to set the stage for the content to follow.

- The **Objectives** comprise two levels: Level I for basic or essential information and Level II for more advanced information. Each instructor must decide what to expect their students to know.
- The **Key Terms** feature alerts students to important terms used in the chapter and found in the glossary.
- The **Background Basics** component alerts students to material that they should have learned or reviewed before starting the chapter. In most cases, these features refer readers to previous chapters to help them find the material if they want to review it.
- The **Overview** gives readers an idea of the chapter content and organization.

- The **Case Study** is a running case feature that first appears at the beginning of a chapter and focuses the students' attention on the subject matter that the chapter covers.
- Appropriate places throughout the chapter provide additional information on the case, such as additional laboratory test results followed by questions that relate to the material presented in preceding sections. The book's website provides the answers to Case Study questions.
- The **Checkpoints** components are integrated throughout the chapter. They are questions that require students to pause along the way to recall or apply information covered in preceding sections. The answers are provided on the book's website.
- A **Summary** concludes the text portion of each chapter to help students bring all the material together.
- **Review Questions** appear at the end of each chapter. The two sets of questions, Level I and Level II, are referenced and organized to correspond to the Level I and Level II objectives. Answers are provided in the Appendix.

The page design features a number of enhancements intended to aid the learning process.

- **Colorful symbols** are used to identify callouts for tables (★) and figures (■) within the chapter text to help students quickly cross-reference from the tables and figures to the text.
- **Figures and tables** are used liberally to help students organize and conceptualize information. This is especially important for visual learners.
- **Microphotographs** are displayed liberally in the book and are typical of those found in a particular disease or disorder. Students should be aware that cell variations occur and that blood and bone marrow findings do not always mimic those found in textbooks. The legend for each microphotograph gives the original magnification but sometimes the image was zoomed to enhance detail.

WHAT'S NEW

Major changes in the text organization are listed here as a quick reference for instructors. In addition to updating, the following changes have been made:

- The leukocyte chapter has been split into two chapters (7 and 8). Chapter 7 includes granulocytes and monocytes; chapter 8 includes lymphocytes.
- An introductory chapter on platelets (Chapter 9) was added to complete the section on blood cells. More detailed information is included in Section Seven, Hemostasis.
- A chapter was added (Chapter 10, The Complete Blood Count and Peripheral Blood Smear Evaluation) to introduce the student to the results and interpretation of two of the most common laboratory tests in hematology.
- Section Five, Hematopoietic Neoplasms, is thoroughly updated using the WHO 2008 classification.
- The body fluid chapter (Chapter 30) was expanded to include more information on procedures and additional body fluids including semen and amniotic fluid. Additional photos of cells are available on the chapter's website.
- Automation in hemostasis testing was moved to the chapter about hemostasis procedures (Chapter 36).
- Chapter 39 includes automation in the hematology laboratory.
- Appendix A contains the answers to chapter review questions. The answers to the case study questions and checkpoints are available on the website.
- Two new comprehensive tables were added to the appendices. The table in Appendix B was developed through a collaborative effort of several authors. It lists hematopoietic neoplasms with the following information on each: immunophenotype using CD markers, cytogenetic abnormalities, and genotypic findings. This table provides a ready reference for information from the chapters in Section Five (Neoplastic Hematologic Disorders) and Section 8 (Hematology Procedures). The table in Appendix C is a comprehensive classification of hematopoietic, lymphopoietic, and histiocytic/dendritic neoplasms using the 2008 WHO classification system.

A COMPLETE TEACHING AND LEARNING PACKAGE

A variety of ancillary materials designed to help instructors be more efficient and effective and students more successful complements this book.

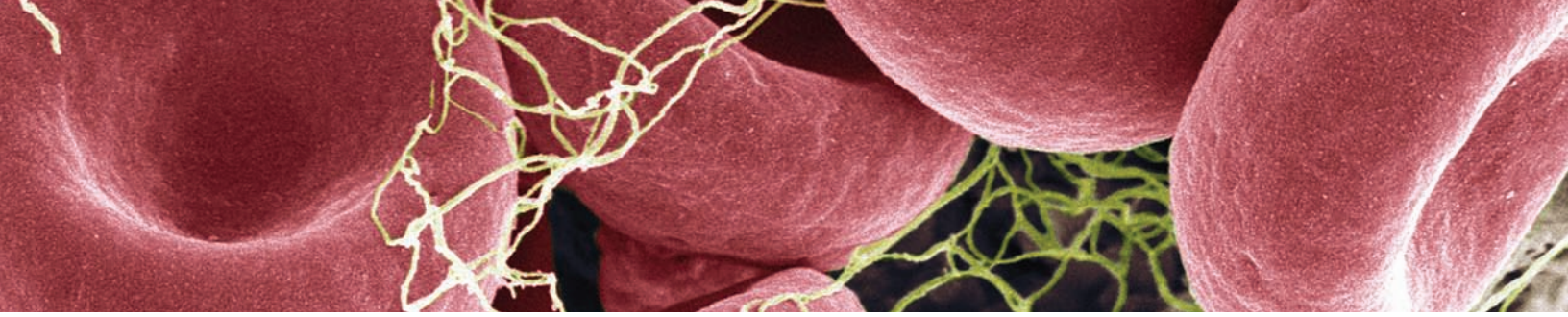
An **Instructor's Resource Center** is available upon adoption of the text and gives the instructor access to a number of powerful tools in an electronic format. The following materials are downloadable:

- The **MyTest** feature includes questions to allow instructors to design customized quizzes and exams. The MyTest guides instructors through the steps to create a simple test with drag-and-drop or point-and-click transfer. Test questions are available either manually or randomly and use online spell checking and other tools to quickly polish the test content and presentation. Instructors can save their tests in a variety of formats both local and network, print as many as 25 variations of a single test, and publish the tests in an online course.
- The **PowerPoint Lectures** tool contains key discussion points and color images for each chapter. This feature provides dynamic, fully designed, integrated lectures that are ready to use, allowing instructors to customize the materials to meet their specific course needs. These ready-made lectures will save instructors time and allow an easy transition into using *Clinical Laboratory Hematology*.
- The **Image Library** feature contains all of the images from the text. Instructors have permission to copy and paste these images into PowerPoint lectures, printed documents, or website as long as they are using *Clinical Laboratory Hematology* as their course textbook.

- The **Instructor's Resource Manual** tool in PDF and Word formats can be accessed.
- The **Bonus Image Library** feature contains microphotographs of normal and abnormal blood cells filed by chapter. These can be downloaded into instructors' digital presentations or used on password-protected course websites.

COMPANION RESOURCES (WWW.PEARSONHIGHERED.COM/HEALTHPROFESSIONSRESOURCES)

This online resource page is completely unique to the market. The website presents additional figures, tables, and information for readers. For procedure chapters, the website includes detailed laboratory procedures that can be adapted and printed for use in the laboratory.



Acknowledgments

Writing a textbook is a complicated task that requires a team of dedicated authors, editors, copy editors, artists, permission researchers, educators, practitioners, content reviewers, project and program managers, and many other individuals behind the scenes. The team that Pearson and the editors put together to make the third edition of this book an excellent hematology and hemostasis resource for students and health care practitioners worked tirelessly over several years to bring the project to completion. The new and returning authors ensured that their chapters were up to date and accurate. Content reviewers and users of the second edition provided helpful suggestions that were incorporated into the chapters. Dr. Brooke Solberg had an important role in reviewing the body fluid chapter and making recommendations that enhanced the chapter's content and organization. We offer our thanks to this group who ensured a quality textbook for a wide audience.

Andrea Klingler was our daily contact who kept us on track even though it meant multiple deadline revisions. She was understanding when our mistakes meant more work for her. Her gentle prodding was evident and appreciated. Her editing was superb.

Rebecca Lazure came into the picture later in the process and played an important role in final copyediting. Patty Gutierrez was instrumental in working with permission researchers to obtain permission for use of copyright works.

John Goucher started the ball moving on the third edition. He had faith in us and provided support and encouragement for another edition of *Clinical Laboratory Hematology*. Jonathan Cheung and Nicole Rangonese were essential in finding authors for support materials including PowerPoints, test questions, and the instructor's guide. This group of author educators contributed behind the scenes to enhance the instructors' use of this book.

A very special thanks goes to Dr. Kristin Landis-Piwowar, Consulting Editor, who accepted a critical editing role late in our process. Her knowledge and expertise in molecular diagnostics proved invaluable. Her attention to detail, writing ability, and suggestions for organization are evident in her editing. Most notably, she was always willing and able to take additional tasks to help keep us on track.

Although he wasn't involved in producing this edition, Mark Cohen was responsible for the creation of the first edition of this text. His keen insights into developing a unique textbook design with pedagogical enhancements has helped *Clinical Laboratory Hematology* become a leading textbook in the field of clinical laboratory science.

Thank you, Pearson, for having faith in us to publish a third edition. Thank you for providing the special team of experts to help us accomplish this task. We recognize that the job is not over but will require the efforts of sales and marketing to ensure widespread use and adoption.

SBM and JLW

The reason I took the task of writing my first hematology textbook was that as an instructor for medical laboratory science students, I could not find a suitable text for them. Thus, my former students were the inspiration for this book. Thank you for your feedback to help make each edition better.

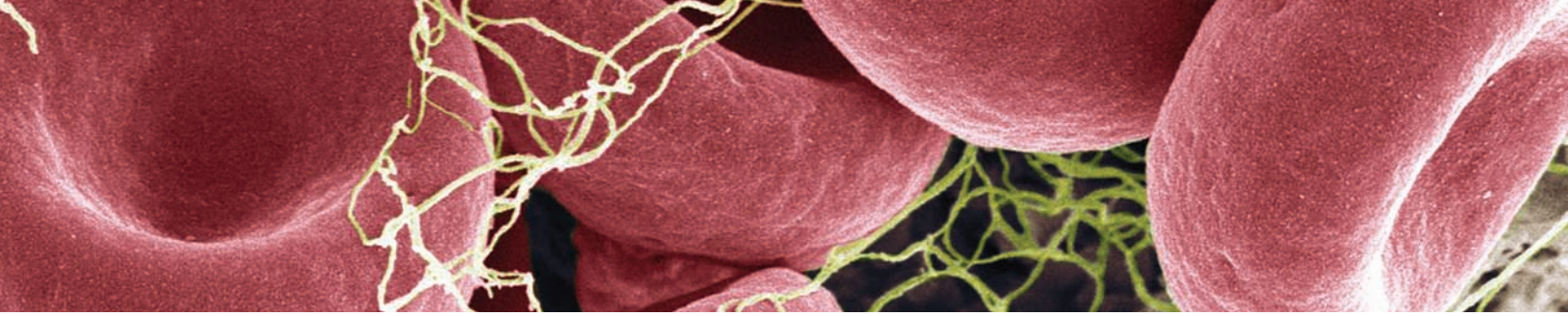
Writing and editing a text of this size is a monumental job. I am privileged to work with my brilliant fellow coeditor and friend, Dr. J. Lynne Williams, who spent many hours of research on topics before editing to ensure that the chapters are up to date and accurate. Her ability to recognize errors is without equal. She spent many late hours at the office to complete editing tasks. We have similar philosophies about teaching hematology and often discussed how to best present the information in this book.

During the time this book was under development, my professional life took over many hours of my personal life. Many thanks to my husband and best friend for his support, sacrifices, and understanding during some very stressful times so this book could become a reality. My parents, George and Helen Olson, instilled in me the confidence that I could accomplish anything I set my heart to. This mind-set has stuck with me through life, especially in this task. I hope that through example I have provided the same to my children and grandchildren.

SBM

I extend a special thank you to my colleagues in the Biomedical Diagnostic and Therapeutic Sciences program at Oakland University—Dr. Kristin Landis-Piwowar, Dr. Sumit Dinda, Lisa DeCeuninck, and our many part-time instructors—who kept the programs moving forward while I was working on this new edition—and to the BDTS students of the past 2 years who tolerated a distracted and often absent-minded professor. To all my former students: You have been my inspiration to try to create a meaningful and useful book to support your educational endeavors. But especially to my coeditor, Dr. Shirlyn McKenzie: thank you for the privilege of accompanying you on this wonderful journey.

JLW



Reviewers

THIRD EDITION

Danyel Anderson, MPH, MT(ASCP)
Ozarks Technical Community College
Springfield, Missouri

Nancy Beamon, MT, BB(ASCP), MT(AAB), AHI(AMT)
Darton State College
Albany, Georgia

Annette Bednar, MSE, MT(ASCP)
Arkansas State University
Jonesboro, Arkansas

Mary Breci, MAT, MT(ASCP)
Midlands Technical College
Columbia, South Carolina

Keri Brophy-Martinez, MHA/ED, BS(ASCP)
Austin Community College
Cedar Park, Texas

Susan Conforti, EdD, MT(ASCP)SBB
Farmingdale State College
Farmingdale, New York

Lorraine Doucette, MS, MLS(ASCP)^{CM}
Anne Arundel Community College
Arnold, Maryland

Kathleen Givens, MSA, MT(ASCP)
Baker College of Allen Park
Allen Park, Michigan

Karen Golemboski, MT(ASCP)
Bellarmine University
Louisville, Kentucky

Candice Grayson, MA, MLS(ASCP)^{CM}
Community College of Baltimore County
Catonsville, Maryland

Virginia Haynes, MT(ASCP)
Lake Superior College
Duluth, Minnesota

Dorilyn J. Hitchcock, MT(ASCP)
University of Central Florida
Orlando, Florida

Lori Howard, MLFSC, BSMT(ASCP)SM
Halifax Community College
Weldon, North Carolina

Phyllis Ingham, EdD
West Georgia Technical College
Douglasville, Georgia

Steve Johnson, MS, MT(ASCP)
Saint Vincent Health Center School of Medical Technology
Erie, Pennsylvania

Kathy Jones, MS, MLS(ASCP)^{CM}
Auburn University at Montgomery
Montgomery, Alabama

Floyd Josephat, EdD, MT(ASCP)
Armstrong Atlantic State University
Savannah, Georgia

Jeff Josifek, CLS (NCA), MLS(ASCP)
Portland Community College
Portland, Oregon

Amy Kapanka, MS, MT(ASCP)SC
Hawkeye Community College
Waterloo, Iowa

Gideon Labiner, MS, MLS(ASCP)^{CM}
University of Cincinnati
Cincinnati, Ohio

Karen Lazarus, SH(ASCP)
Youngstown State University
Youngstown, Ohio

John Scariano, PhD, MLS(ASCP)
University of New Mexico
Albuquerque, New Mexico

Pam St. Laurent, EdD, MT(ASCP)
Florida Gulf Coast University
Fort Myers, Florida

Mary Ellen Tancred, MLS(ASCP), SH(ASCP)
Columbus State Community College
Columbus, Ohio

Elyse Wheeler, MT(ASCP)
Albany College of Pharmacy and Health Sciences
Albany, New York

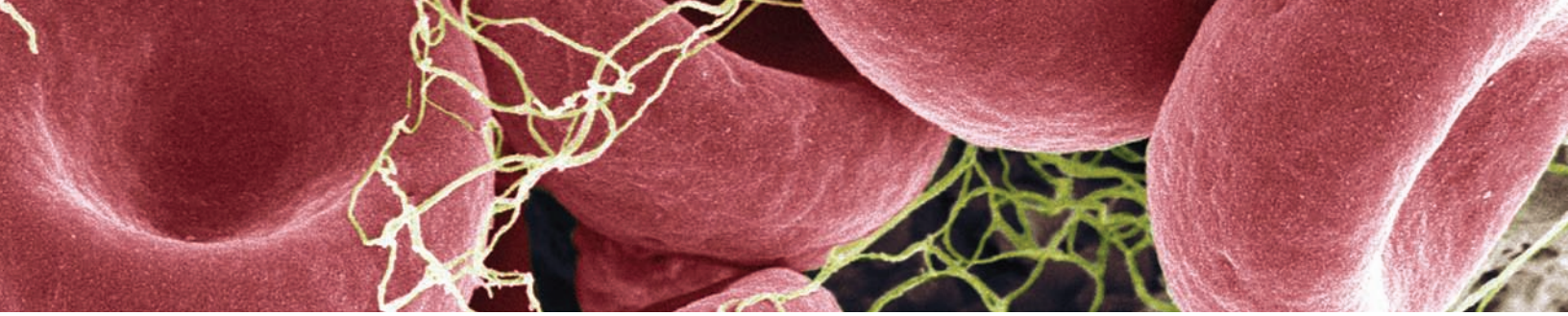
Joan Young, MHA, MT(ASCP)
Southwest Wisconsin Technical College
Fennimore, Wisconsin

CONTENT-LEVEL REVIEW PANEL

Linda L. Breiwick, BS, CLS(NCA), MT(ASCP)
Program Director, Medical Laboratory Technology
Shoreline Community College
Seattle, Washington

Linda Comeaux, BS, CLS(NCA)
Vice-President for Instruction
Red Rocks Community College
Lakewood CO

Mona Gleysteen, MS, CLS(NCA)
Program Director, Medical Laboratory Technician Program
Lake Area Technical Institute
Watertown, South Dakota



Contributors

Sue S. Beglinger, MS, MLS(ASCP)
Program Director, Medical Laboratory Technology
School of Health Sciences
Madison Area Technical College
Madison, WI
Chapter 22

Gail Bentley, MD
Hematopathologist, Associate Professor
Department of Pathology
Detroit Medical Center
Detroit, MI
Chapters 26, 27

Cheryl Burns, MS, MLS(ASCP)CM
Associate Professor, Department of Clinical Laboratory Sciences
University of Texas Health Science Center at San Antonio
San Antonio, TX
Chapters 37, 39, 43

Diana L. Cochran-Black, Dr PH, MLS(ASCP)CM, SH(ASCP)CM
Associate Professor, Department of Medical Laboratory Sciences
Wichita State University
Wichita, Kansas
Chapter 17

Fiona E. Craig, MD
Professor, Hematopathology
Department of Pathology
University of Pittsburgh School of Medicine
Pittsburgh, PA
Chapters 28, 40

Mary Ann Dotson, BS MLS(ASCP)
Analytical Specialist–Hematology
Duke University Health System
Chapter 37

Aamir Ehsan, MD
Chief, Pathology and Laboratory Medicine
South Texas Veterans Health Care System
Associate Professor, Department of Pathology
University of Texas Health Science Center at San Antonio
San Antonio, TX
Chapters 29, 38

Sandra Estelle, MT(ASCP)SH
Laboratory Operations Supervisor
Children's Healthcare of Atlanta
Atlanta, GA
Chapter 36

Kathleen Finnegan, MS, MT(ASCP), SHCM
Department of Clinical Laboratory Sciences
Stony Brook University
Stony Brook, NY
Chapter 8

Ali Gabali, MD, PhD
Assistant Professor, School of Medicine
Wayne State University
Director, Coagulation; Director, Flow Cytometry
Department of Pathology
Detroit Medical Center and Karmanos Cancer Center
Detroit, MI
Chapter 34

Joel D. Hubbard, PhD, MLS(ASCP)
Associate Professor, Laboratory Sciences and Primary Care
Texas Tech University Health Science Center
Lubbock, TX
Chapters 5, 15

Beverly Kirby, EdD, MLS(ASCP)
Associate Professor, Division of Medical Laboratory Sciences
School of Medicine
West Virginia University
Morgantown, WV
Chapter 34

Martha Lake, EdD, MLS(ASCP)
Professor Emeritus
West Virginia University
Morgantown, WV
Chapter 18

Rebecca J. Laudicina, PhD, MLS(ASCP)CM
Professor Emeritus
Division of Clinical Laboratory Science
University of North Carolina at Chapel Hill
Chapel Hill, NC
Chapters 13, 16

Louann W. Lawrence, DrPH, MLS (ASCP)
Professor Emeritus
Clinical Laboratory Sciences
Louisiana State University Health Sciences Center
New Orleans, LA
Adjunct Faculty
Medical Laboratory Sciences
Tarleton State University
Fort Worth, TX
Chapter 25

John H. Landis, MS, MLS(ASCP)
Professor Emeritus, Ferris State University
Canadian Lakes, Michigan
Adjunct Professor, University of Cincinnati
Adjunct Professor, University of North Florida
Chapter 10

Kristin Landis-Piwowar, PhD, MLS(ASCP)^{CM}
Biomedical Diagnostic and Therapeutic Sciences
School of Health Sciences
Oakland University
Rochester, MI
Chapters 7, 10, 21

Susan Leclair, PhD, MT(ASCP)
Chancellor Professor, Medical Laboratory Science
University of Massachusetts Dartmouth
Dartmouth, MA
Chapters 26, 27

Sally Lewis, PhD, MLS(ASCP)^{CM}, MB^{CM}, HTL^{CM}
Department Head and Associate Professor
Department of Medical Laboratory Sciences
Tarleton State University
Fort Worth, TX
Chapter 29, 42

David L. McGlasson, MS, MLS(ASCP)^{CM}
Clinical Research Scientist
59th Clinical Research Division
Laboratory Services
JBSA Lackland, TX
Chapter 36

Shirlyn B. McKenzie, PhD, MLS(ASCP)^{CM}, SH^{CM}
Distinguished Teaching Professor
Professor Emeritus and Chair Emeritus
Department of Clinical Laboratory Sciences
University of Texas Health Science Center at San Antonio
San Antonio, TX
Chapters 1, 6, 11, 12, 23

Lucia E. More, Col, USAF, BSC, MS, MT(ASCP)
Commander, 959th Clinical Support Squadron
JBSA Fort Sam Houston, TX
Chapter 43

Barbara O'Malley, MD, FASCP, MT(ASCP)
Medical Director, Clinical Laboratories
Department of Pathology
Harper University Hospital
Detroit, MI
Chapters 31, 33

Catherine N. Otto, PhD, MBA, MLS(ASCP)^{CM}
Associate Professor
Medical Laboratory Science
Salisbury University
Salisbury, MD
Chapter 11

Keila Poulsen, BS, MLS(ASCP)^{CM}, H, SH
Hematology and Histology Supervisor
Eastern Idaho Regional Medical Center
Idaho Falls, ID
Chapter 10

Tim R. Randolph, PhD, MT(ASCP)
Chair and Associate Professor
Department of Clinical Laboratory Science
Saint Louis University
St. Louis, MO
Chapters 14, 24

Stacey Robinson, MS, MLS(ASCP)^{CM}, SH^{CM}
Hematology Supervisor, Clinical Laboratory
United States Army Medical Research Institute of Infectious Diseases
Fort Detrick, MD
Chapters 5, 15

Annette Schlueter, MD, PhD
Associate Professor
Department of Pathology
University of Iowa
Iowa City, IA
Chapter 3

Linda Smith, PhD, MLS(ASCP)^{CM}, BB
Professor and Chair
Department of Clinical Laboratory Sciences
University of Texas Health Science Center at San Antonio
San Antonio, TX
Chapters 19, 20

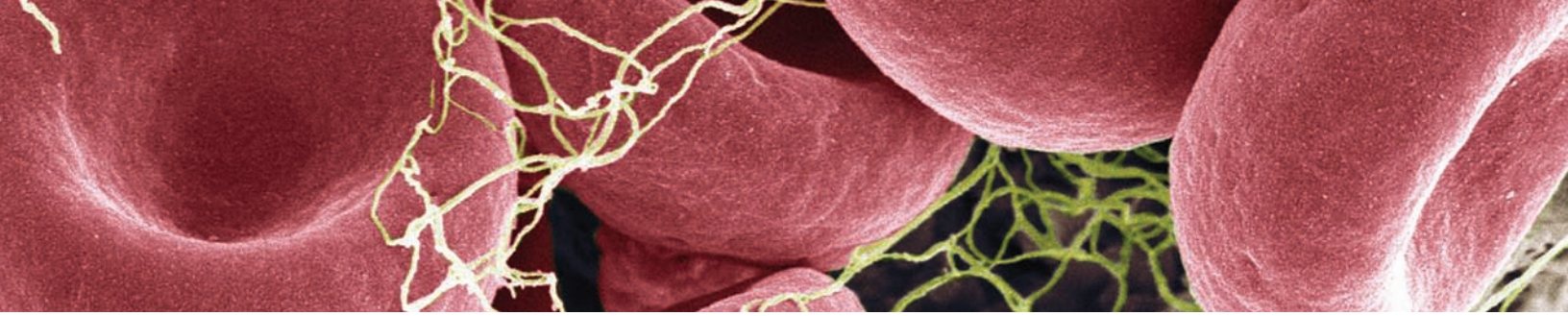
Sara Taylor, PhD, MLS(ASCP), MB^{CM}
Associate Professor
Medical Laboratory Sciences
Tarleton State University
Fort Worth, TX
Chapters 25, 29, 42

Jerelyn Walters, MLS(ASCP), SH
Technical Supervisor, Esoteric Testing
ACL Laboratories
Milwaukee, WI
Chapter 30

J. Lynne Williams, PhD, MT(ASCP)
Professor and Director
Biomedical Diagnostic and Therapeutic Sciences
School of Health Sciences
Oakland University
Rochester, MI
Chapters 2, 4, 8, 9, 32, 35

Kathleen S. Wilson, MD, FCAP, FACMG
Professor, Department of Pathology and The McDermott Center for Human Growth and Development
Director, Cytogenomic Microarray Analysis Laboratory
University of Texas Southwestern Medical Center
Dallas, TX
Chapter 41

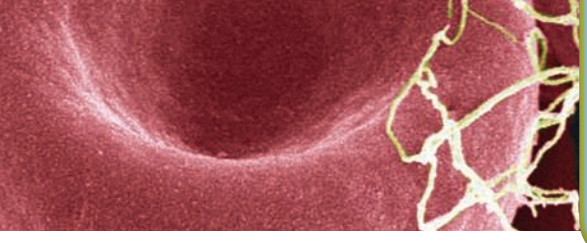
Andrea C. Yunes, MD
Audie L. Murphy Memorial VA Hospital
San Antonio, TX
Chapter 29



Select Abbreviations Used

AHG	Antihuman globulin
AIDS	Acquired immune deficiency syndrome
AIHA	Autoimmune hemolytic anemia
AL	Acute leukemia
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANLL	Acute nonlymphocytic leukemia
APTT	Activated partial thromboplastin time
ARC	AIDS-related complex
Band	Nonsegmented neutrophil
BCR	B-cell receptor
BT	Bleeding time
CBC	Complete blood count
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CEL, NOS	Chronic eosinophilic leukemia, not otherwise specified
CFU	Colony-forming unit
CGL	Chronic granulocytic leukemia
CHr	Reticulocyte hemoglobin
CHCMr	Mean corpuscular hemoglobin concentration of the reticulocyte
CKI	Cyclin-dependent kinase inhibitor
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid (myelogenous) leukemia
CMML	Chronic myelomonocytic leukemia
CMV	Cytomegalovirus
CNL	Chronic neutrophilic leukemia
DAF	Decay-accelerating factor
DAT	Direct antiglobulin test
DIC	Disseminated intravascular coagulation
dL	Deciliter
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
EBV	Epstein-Barr virus
EPO	Erythropoietin
ER	Endoplasmic reticulum
ET	Essential thrombocythemia
FA	Fanconi's anemia
FAB	French-American-British
FFP	Fresh frozen plasma
G6PD	Glucose-6-phosphate dehydrogenase
GMP	Granulocyte/monocytes progenitor
Hb	Hemoglobin
Hct	Hematocrit
HDFN	Hemolytic disease of the fetus and newborn
HES	Hypereosinophilic syndrome
HPFH	Hereditary persistence of fetal hemoglobin
HUS	Hemolytic uremic syndrome
IAT	Indirect antiglobulin test
Ig	Immunoglobulin
INR	International normalized ratio
IRF	Immature reticulocyte fraction
ISC	Irreversibly sickled cell
ISI	International sensitivity index
ITP	Immune thrombocytopenia also called Idiopathic thrombocytopenic purpura
L	Liter
LAP	Leukocyte alkaline phosphatase
LCAT	Lecithin-cholesterol acyl transferase
LD	Lactic dehydrogenase
Lymph	Lymphocyte
MAHA	Microangiopathic hemolytic anemia
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular (cell) volume
MDS	Myelodysplastic syndrome
MEP	megakaryocytic/erythroid progenitor
MHC	Major histocompatibility complex
mcG	Microgram
mcL	Microliter
mcM	Micrometer
mL	Milliliter
Mono	Monocyte
MPD	Myeloproliferative disorder
MPN	Myeloproliferative neoplasm
MW	Molecular weight
NRBC	Nucleated red blood cell
PAS	Periodic acid-Schiff

PCH	Paroxysmal cold hemoglobinuria
PCR	Polymerase chain reaction
PDW	Platelet distribution width
PIVKA	Protein induced by vitamin-K absence (or antagonist)
PK	Pyruvate kinase
PMN	Polymorphonuclear neutrophil
PNH	Paroxysmal nocturnal hemoglobinuria
PT	Prothrombin time
RA	Refractory anemia
RB	Retinoblastoma
RAEB	Refractory anemia with excess blasts
RARS	Refractory anemia with ring sideroblasts
RBC	Red blood cell
RDW	Red cell distribution width
RER	Rough endoplasmic reticulum
RET-He	Reticulocyte hemoglobin content measured by Sysmex instrument
rHuEPO	Recombinant human erythropoietin
RNA	Ribonucleic acid
RPI	Reticulocyte production index
SCIDS	Severe combined immunodeficiency syndrome
Seg	Segmented neutrophil
SER	Smooth endoplasmic reticulum
SLL	Small lymphocytic lymphoma
TCR	T-cell receptor
TF	Transcription Factor
TIBC	Total iron-binding capacity
TPO	Thrombopoietin
TRAP	Tartrate-resistant acid phosphatase
TTP	Thrombotic thrombocytopenic purpura
UTR	Untranslated region
VWF	von Willebrand factor
WBC	White blood cell
WHO	World Health Organization



1 Introduction

SHIRLYN B. McKENZIE, PhD

Objectives—Level I and Level II

At the end of this unit of study, the student should be able to:

1. Compare the reference intervals for hemoglobin, hematocrit, erythrocytes, and leukocytes in infants, children, and adults.
2. Identify the function of erythrocytes, leukocytes, and platelets.
3. Describe the composition of blood.
4. Explain the causes of change in the steady state of blood components.
5. Describe reflex testing, and identify the laboratory's role in designing reflex testing protocols.
6. Define hemostasis and describe the result of an upset in the hemostatic process.
7. Identify hematology and hemostasis screening tests.
8. List the three components of laboratory testing and correlate errors with each component.

Key Terms

Activated partial thromboplastin time (APTT)
Complete blood count (CBC)
Diapedese
Erythrocyte
Hematocrit
Hematology
Hematopoiesis
Hemoglobin
Hemostasis
Leukocyte
Plasma
Platelet
Prothrombin time (PT)
RBC index
Red blood cell (RBC)
Reflex testing
Thrombocyte
White blood cell (WBC)

Background Basics

Students should complete courses in biology and physiology before beginning this study of hematology.

Chapter Outline

CASE STUDY

We will address this case study throughout the chapter.

Aaron, a 2-year-old male, was seen by his pediatrician because he had a fever of 102 to 104°F over the past 24 hours. Aaron was lethargic. Before this, he had been in good health except for two episodes of otitis.

Consider why the pediatrician might order laboratory tests and how this child's condition might affect the composition of his blood.

OVERVIEW

Hematology is the study of blood and blood-forming organs. The hematology laboratory is one of the busiest areas of the clinical laboratory. Even small, limited-service laboratories usually offer hematology tests. This chapter is an introduction to the composition of blood and the testing performed in the hematology laboratory to identify the presence and cause of disease.

INTRODUCTION

Blood has been considered the essence of life for centuries. One of the Hippocratic writings from about 400 B.C. describes the body as being a composite of four humors: black bile, blood, phlegm, and yellow bile. It is thought that the theory of the four humors came from the observation that four distinct layers form as blood clots in vitro: a dark-red, almost black, jellylike clot (black bile); a thin layer of oxygenated red cells (blood); a layer of white cells and platelets (phlegm); and a layer of yellowish serum (yellow bile).[1] Health and disease were thought to occur as a result of an upset in the equilibrium of these humors.

The cellular composition of blood was not recognized until the invention of the microscope. With the help of a crude magnifying device that consisted of a biconvex lens, Leeuwenhoek (1632–1723) accurately described and measured the **red blood cells** (also known as **RBCs** or **erythrocytes**). The discovery of **white blood cells** (also known as **WBCs** or **leukocytes**) and **platelets** (also known as **thrombocytes**) followed after microscope lenses were improved.

As a supplement to these categorical observations of blood cells, Karl Vierordt in 1852 published the first quantitative results of blood cell analysis.[2] His procedures for quantification were tedious and time consuming. After several years, many others attempted to correlate blood cell counts with various disease states.

Improved methods of blood examination in the 1920s and the increased knowledge of blood physiology and blood-forming organs in the 1930s allowed anemias and other blood disorders to be studied on a rational basis. In some cases, the pathophysiology of hematopoietic disorders was realized only after the patient responded to experimental therapy.

Contrary to early hematologists, modern hematologists recognize that alterations in the components of blood are the *result* of disease, not a *primary cause* of it. Under normal conditions, the production of blood cells in the bone marrow, their release to the peripheral blood, and their survival are highly regulated to maintain a steady state of morphologically normal cells. Quantitative and qualitative hematologic abnormalities can result when an imbalance occurs in this steady state.

COMPOSITION OF BLOOD

Blood is composed of a liquid called **plasma** and of cellular elements, including leukocytes, platelets, and erythrocytes. The normal adult has about 6 liters of this vital fluid, which composes from 7% to 8% of the total body weight. Plasma makes up about 55% of the blood volume; about 45% of the volume is composed of erythrocytes, and 1% of the volume is composed of leukocytes and platelets. Variations in the quantity of these blood elements are often the first sign of disease occurring in body tissue. Changes in diseased tissue may be detected by laboratory tests that measure deviations from normal in blood constituents. Hematology is primarily the study of the formed cellular blood elements.

The principal component of plasma is water, which contains dissolved ions, proteins, carbohydrates, fats, hormones, vitamins, and enzymes. The principal ions necessary for normal cell function include calcium, sodium, potassium, chloride, magnesium, and hydrogen. The main protein constituent of plasma is albumin, which is the most important component in maintaining osmotic pressure. Albumin also acts as a carrier molecule, transporting compounds such as bilirubin and heme. Other blood proteins carry vitamins, minerals, and lipids. Immunoglobulins, synthesized by lymphocytes, and complement are specialized blood proteins involved in immune defense. The coagulation proteins responsible for **hemostasis** (arrest of bleeding) circulate in the blood as inactive enzymes until they are needed for the coagulation process. An upset in the balance of these dissolved plasma constituents can indicate a disease in other body tissues.

Blood plasma also acts as a transport medium for cell nutrients and metabolites; for example, the blood transports hormones manufactured in one tissue to target tissue in other parts of the body. Albumin transports bilirubin, the main catabolic residue of hemoglobin, from the spleen to the liver for excretion. Blood urea nitrogen, a nitrogenous waste product, is carried to the kidneys for filtration and excretion. Increased concentration of these normal catabolites can indicate either increased cellular metabolism or a defect in the organ responsible for their excretion. For example, in liver disease, the bilirubin level in blood increases because the liver is unable to function normally and clear the bilirubin. In hemolytic anemia, however, the bilirubin concentration can rise because of the increased metabolism of hemoglobin that exceeds the ability of a normal liver to clear bilirubin.

When body cells die, they release their cellular constituents into surrounding tissue. Eventually, some of these constituents reach the blood. Many constituents of body cells are specific for the cell's particular function; thus, increased concentration of these constituents in the blood, especially enzymes, can indicate abnormal cell destruction in a specific organ.

Blood cells are produced and develop in the bone marrow. This process is known as **hematopoiesis**. Undifferentiated hematopoietic stem cells (precursor cells) proliferate and differentiate under the influence of proteins that affect their function (cytokines). When the cell reaches maturity, it is released into the peripheral blood.

Each of the three cellular constituents of blood has specific functions. Erythrocytes contain the vital protein **hemoglobin**, which is responsible for transport of oxygen and carbon dioxide between the lungs and body tissues. The five major types of leukocytes are neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Each type of leukocyte has a role in defending the body against foreign

pathogens such as bacteria and viruses. Platelets are necessary for maintaining hemostasis. Blood cells circulate through blood vessels, which are distributed throughout every body tissue. Erythrocytes and platelets generally carry out their functions without leaving the vessels, but leukocytes **diapedese** (pass through intact vessel walls) to tissues where they defend against invading foreign pathogens.

CASE STUDY (continued from page 2)

1. If Aaron was diagnosed with otitis media, what cellular component(s) in his blood would be playing a central role in fighting this infection?

REFERENCE INTERVALS FOR BLOOD CELL CONCENTRATION

Physiologic differences in the concentration of cellular elements can occur according to race, age, sex, and geographic location; pathologic changes in specific blood cell concentrations can occur as the result of disease or injury. The greatest differences in reference intervals occur between newborns and adults. In general, newborns have a higher erythrocyte concentration than any other age group. The erythrocytes are also larger than those of adults. In the 6 months after birth, erythrocytes gradually decrease in number and then slowly increase. Hemoglobin and erythrocyte counts increase in children between the ages of 5 and 17. The leukocyte concentration is high at birth but decreases after the first year of life. A common finding in young children is an absolute and relative lymphocytosis (increase in lymphocytes). After puberty, males have higher hemoglobin, **hematocrit** (packed red blood cell volume in whole blood), and erythrocyte levels than females. The hemoglobin level decreases slightly after age 70 in males. This is thought to be due to the decrease in testosterone. Tables A through K on the inside covers of this text give hematologic reference intervals for various age groups and by sex if appropriate.

Each individual laboratory must determine reference intervals of hematologic values to account for the physiologic differences of a population in a specific geographical area. Reference intervals for a hematologic parameter are determined by calculating the mean ±2 standard deviations for a group of healthy individuals. This interval represents the reference interval for 95% of normal individuals. A value just below or just above this interval is not necessarily abnormal; normal and abnormal overlap. Statistical probability indicates that about 5% of normal individuals will fall outside the ±2 standard deviation range. The further a value falls from the reference interval, however, the more likely the value is to be abnormal.

CASE STUDY

Aaron's physician ordered a complete blood count (CBC). The results are Hb 11.5 g/dL (115 g/L); Hct 34% (0.34 L/L).

2. What parameters, if any, are outside the reference intervals? Why do you have to take Aaron's age into account when evaluating these results?

HEMOSTASIS

Hemostasis is the property of the circulation that maintains blood as a fluid within the blood vessels and the system's ability to form a barrier (blood clot) to prevent excessive blood loss when the vessel is traumatized, limit the barrier to the site of injury, and dissolve the clot to ensure normal blood flow when the vessel is repaired. Hemostasis occurs in stages called *primary* and *secondary hemostasis* and *fibrinolysis* (breakdown of fibrin). These stages are the result of interaction of platelets, blood vessels, and proteins circulating in the blood. An upset in any of the stages can result in bleeding or abnormal blood clotting (thrombosis). Laboratory testing for abnormalities in hemostasis is usually performed in the hematology section of the laboratory; occasionally, hemostasis testing is performed in a separate specialized section of the laboratory.

☑ CHECKPOINT 1-1

What cellular component of blood can be involved in disorders of hemostasis?

BLOOD COMPONENT THERAPY

Blood components can be used in therapy for various hematologic and nonhematologic disorders. Whole blood collected from donors can be separated into various cellular and fluid components. Only the specific blood component (i.e., platelets for thrombocytopenia or erythrocytes for anemia) needed by the patient will be administered. In addition, the components can be specially prepared for the patient's specific needs (i.e., washed erythrocytes for patients with IgA deficiency to reduce the risk of anaphylactic reactions). Table 1-1 ★ lists the various components that can be prepared for specific uses. The reader may want to refer back to this table when reading subsequent chapters about therapies that use these components.

LABORATORY TESTING IN THE INVESTIGATION OF A HEMATOLOGIC PROBLEM

Laboratory testing is divided into three components: pre-examination, examination, and post-examination (formerly known as preanalytical, analytical, postanalytical). The *pre-examination* component includes all aspects that occur prior to the testing procedure that affect the test outcome such as phlebotomy technique and storage of the specimen after it is drawn but before the test is run. The *examination phase* refers to all aspects affecting the test procedure. The *post-examination* component includes all aspects after the testing is completed such as reporting of results. These three aspects of testing are the backbone of a quality assessment program. See Chapters 10 and 43 for a detailed description of these three phases.

A physician's investigation of a hematologic problem includes taking a medical history and performing a physical examination. Clues provided by this preliminary investigation help guide the physician's choice of laboratory tests to help confirm the diagnosis. The challenge is to select appropriate tests that contribute to a cost-effective and efficient diagnosis. Laboratory testing usually begins

★ **TABLE 1-1** Blood Components and Their Uses

Component Name	Composition	Primary Use
Whole blood	Red blood cells and plasma	Not used routinely; can be used in selected trauma, autologous transfusions, and neonatal situations; increases oxygen-carrying capacity and volume
Packed red blood cells (PRBCs)	PRBCs	Used in individuals with symptomatic anemia to increase oxygen-carrying capability
PRBCs, washed	PRBCs; plasma and most leukocytes and platelets removed	Used for individuals with repeated allergic reactions to components containing plasma and for IgA-deficient individuals with anaphylactic reactions to products containing plasma
PRBCs, leukoreduced	PRBCs; WBC removed	Used to decrease the risk of febrile nonhemolytic transfusion reaction, HLA sensitization, and cytomegalovirus (CMV) transmission
PRBCs, frozen, deglycerolized	PRBCs frozen in cryoprotective agent, thawed, washed	Used for individuals with rare blood groups (autologous donation)
PRBCs, irradiated	PRBCs with lymphocytes inactivated	Used to reduce the risk of graft-vs-host disease
Platelets, pooled[a]	4–6 units of random donor platelets	Used to increase platelet count and decrease bleeding when there is a deficiency or abnormal function of platelets
Platelets, single[a] donor (pheresis)	Equivalent of 4–6 donor platelets collected from single donor	Used to treat patients refractory to random platelet transfusion or to increase platelet count due to a deficiency or abnormal function of platelets
Fresh frozen plasma (FFP)	Plasma with all stable and labile coagulation factors; frozen within 8 hours of collection of unit of blood	Used to treat patients with multiple coagulation factor deficiencies; disseminated intravascular coagulation (DIC); used with packed RBC in multiple transfusions
Cryoprecipitated AHF[b]	Concentrated FVIII, fibrinogen, FXIII, von Willebrand factor	Used to treat patients with hypofibrinogenemia, hemophilia A, von Willebrand's disease, FXIII deficiency
Plasma, cryo-poor	Plasma remaining after cryo removed	Used to treat thrombotic thrombocytopenic purpura (TTP)
Liquid plasma	Plasma not frozen within 8 hours of collection	Used in patients with deficiency of stable coagulation factor(s) and for volume replacement
Granulocytes	Granulocytes	Used to treat the neutropenic patient who is septic and unresponsive to antimicrobials and who has chance of marrow recovery

[a] Platelets can also be leukoreduced or irradiated. See PRBC for reasons.
[b] Cryo = Cryoprecipitated antihemophilic factor

Courtesy of Linda Smith, Ph.D., MLS(ASCP)^{CM}; adapted from the *circular of information for the use of human blood and blood components*. Prepared jointly by the American Association of Blood Banks, America's Blood Centers, and the American Red Cross (2002).

with screening tests; based on results of these tests, more specific tests are ordered. The same tests can be ordered again on follow-up to track disease progression, evaluate treatment, identify side effects and complications, or assist in prognosis.

Hematology screening tests include the **complete blood count (CBC)**, which quantifies the white blood cells (WBCs), red blood cells (RBCs), hemoglobin, hematocrit, and platelets and the **RBC indices** (Chapter 10). The indices are calculated from the results of the hemoglobin, RBC count, and hematocrit to define the size and hemoglobin content of RBCs. The indices are important parameters used to differentiate causes of anemia and help direct further testing. The CBC can also include a WBC differential. This procedure enumerates the five types of WBCs and reports each as a percentage of the total WBC count. A differential is especially helpful if the WBC count is abnormal. When the count is abnormal, the differential identifies which cell type is abnormally increased or decreased and determines whether immature and/or abnormal forms are present, thus providing a clue to diagnosis. The morphology of RBCs and platelets is also studied as a routine part of the differential.

If a hemostasis problem is suspected, the screening tests include the platelet count, **prothrombin time (PT)**, and **activated partial thromboplastin time (APTT)** (Chapter 36). The PT and APTT tests involve adding calcium and thromboplastin or partial thromboplastin to a sample of citrated plasma and determining the time it takes to form a clot. These tests provide clues that guide the choice of follow-up tests to help identify the problem.

Follow-up testing that is done based on results of screening tests is referred to as **reflex testing**. These testing protocols are sometimes referred to as *algorithms*. Follow-up tests can include not only hematologic tests but also chemical, immunologic, and/or molecular analysis. As scientists learn more about the pathophysiology and treatment of hematologic disease and hemostasis, the number of tests designed to assist in diagnosis expands. Errors in selection of the most appropriate laboratory tests and interpretation of results can result in misdiagnosis or treatment errors and is a major source of poor patient outcomes. Laboratory professionals can assist in promoting good patient outcomes by assisting physicians and patient care teams in selecting the most efficient and effective testing strategies[3–5] and assisting in interpretation of test results.[6] Readers are urged to use the reflex testing concept in their thought processes when studying the laboratory investigation of a disease.

In an effort to help the student gain the knowledge to perform these functions, in this text, each hematologic disorder is discussed in the following order: pathophysiology (and etiology, if known), clinical findings, laboratory findings, and treatment. The reader should consider which laboratory tests provide the information necessary to identify the cause of the disorder based on the suspected disorder's pathophysiology. Although it is unusual for the physician to provide a patient history or diagnosis to the laboratory when ordering tests, this information is often crucial to direct investigation and assist in interpretation of the test results. In any case, if laboratory professionals need more patient information to perform testing appropriately, they should obtain the patient's chart or call the physician.

☑ CHECKPOINT 1-2

A 13-year-old female saw her physician for complaints of a sore throat, lethargy, and swollen lymph nodes. A CBC was performed with the following results: Hb 9.0 g/dL (90 g/L); Hct 30% (0.30 L/L); WBC 15×10^9/L; (15×10^3/mL). On the basis of these results, should reflex testing be performed?

Summary

Hematology is the study of the cellular components of blood: erythrocytes, leukocytes, and platelets. Physiological changes in the concentrations of these cells occur from infancy until adulthood. Diseases can upset the steady state concentration of these parameters. A CBC is usually performed as a screening test to determine whether there are quantitative abnormalities in blood cells. The physician can order reflex tests if one or more of the CBC parameters are outside the reference interval. Platelet count, PT, and APTT are screening tests for disorders of hemostasis.

Changes in the health care system focus on containing costs while maintaining quality of care. The laboratory's role in this system is to work with physicians to optimize utilization of laboratory testing.

Review Questions

Level I and Level II

1. In which group of individuals would you expect to find the highest reference intervals for hemoglobin, hematocrit, and erythrocyte count? (Objective 1)
   A. newborns
   B. males older than 12 years of age
   C. females older than 17 years of age
   D. children between 1 and 5 years of age
2. Which cells are important in transporting oxygen and carbon dioxide between the lungs and body tissues? (Objective 2)
   A. platelets
   B. leukocytes
   C. thrombocytes
   D. erythrocytes
3. Forty-five percent of the volume of blood is normally composed of: (Objective 3)
   A. erythrocytes
   B. leukocytes
   C. platelets
   D. plasma
4. Alterations in the concentration of blood cells generally are the result of: (Objective 4)
   A. laboratory error
   B. amount of exercise before blood draw
   C. a disease process
   D. variations in analytical equipment
5. Leukocytes are necessary for: (Objective 2)
   A. hemostasis
   B. defense against foreign pathogens
   C. oxygen transport
   D. excretions of cellular metabolites
6. Laboratories can use which type of testing to help direct the physician's selection of appropriate testing after screening tests are performed? (Objective 5)
   A. reflexive based on results of screening tests
   B. manual repeat of abnormal results
   C. second test by a different instrument
   D. standing orders for all inpatients

7. Screening tests used to evaluate the hemostasis system include: (Objective 6)
   A. PT and APTT
   B. CBC
   C. hemoglobin
   D. WBC count

8. A patient blood specimen is stored in a car for 2 hours with the outside temperature of 95°. This is an example of error in which component of testing? (Objective 8)
   A. pre-examination
   B. examination
   C. post-examination

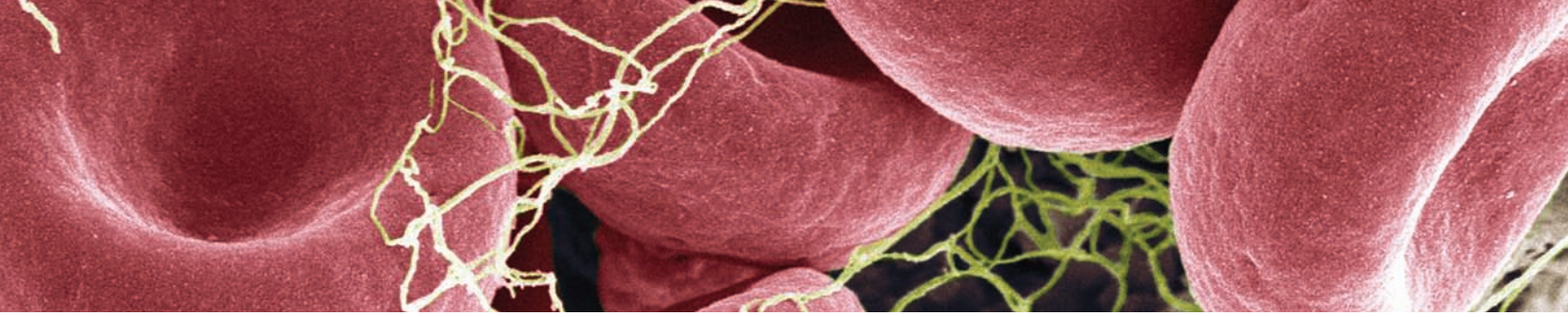
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Wintrobe MM. *Blood: Pure and Eloquent*. New York: McGraw-Hill; 1980.
2. Vierordt K. Zahlungen der Blutkorperchen des Menschen. *Arch Physiol-Heilk*. 1852;11:327.
3. Hernandez JS. Cost-effectiveness of laboratory testing. *Arch Pathol Lab Med*. 2003;127:440–445.
4. From P, Barak M. Cessation of dipstick urinalysis reflex testing and physician ordering behavior. *Am J Clin Pathol*. 2012;137(3):486–489.
5. Feldman LS, Shihab HM, Thiemann D et al. Impact of providing fee data on laboratory test ordering. *JAMA Intern Med*. 2013;173(10):903–908.
6. Dighe AS, Makar RS, Lewandrowski KB. Medicolegal liability in laboratory medicine. *Sem Diag Path*. 2007;24(2):98–107.



2 Cellular Homeostasis

J. LYNNE WILLIAMS, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe the location, morphology, and function of subcellular organelles of a cell.
2. Describe the lipid asymmetry found in the plasma membrane of most hematopoietic cells.
3. Differentiate the parts of the mammalian cell cycle.
4. Define R (*restriction point*) and its role in cell-cycle regulation.
5. Define *apoptosis* and explain its role in normal human physiology.
6. Classify and give examples of the major categories of initiators and inhibitors of apoptosis.
7. List the major events regulated by apoptosis in hematopoiesis.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Explain the significance of SNPs, introns, exons, UTRs, and post-translational protein modifications.
2. List the components and explain the function of the ubiquitin-proteosome system.
3. Define *cyclins* and *Cdks* and their role in cell-cycle regulation; describe the associated Cdk partners and function of cyclins D, E, A, and B.
4. Define *CAK* (Cdk-activating kinase) and the two major classes of CKIs (cyclin-dependent kinase inhibitors) and describe their function.
5. Compare the function of cell-cycle checkpoints in cell-cycle regulation.
6. Describe/illustrate the roles of p53 and pRb in cell-cycle regulation.
7. Propose how abnormalities of cell-cycle regulatory mechanisms can lead to malignancy.
8. Define *caspases* and explain their role in apoptosis.
9. Differentiate the extrinsic and intrinsic pathways of cellular apoptosis.
10. Define and contrast the roles of pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

11. Describe apoptotic regulatory mechanisms.
12. Give examples of diseases associated with increased apoptosis and inhibited (decreased) apoptosis.
13. Define *epigenetics*, and give examples of epigenetic changes associated with gene silencing.
14. Differentiate, using morphologic observations, the processes of necrotic cell death and apoptotic cell death.

Key Terms

Anti-oncogene/tumor suppressor gene
Apoptosis
Caspase
Cell-cycle checkpoint
Cyclins/Cdk
Epigenetics
Exon
Genome/genomics
Intron
Mutation
Necrosis
Polymorphism
Post-translational modification
Proteomics
Proteosome
Proto-oncogene
Quiescence (G_0)
Restriction point (R)
Single nucleotide polymorphism (SNP)
Tissue homeostasis
Transcription factor (TF)
Ubiquitin
Untranslated region (UTR)

Background Basics

LEVEL I AND LEVEL II

Students should have a solid foundation in basic cell biology principles, including the component parts of a cell and the structure and function of cytoplasmic organelles. They should have an understanding of the segments composing a cell cycle (interphase and mitosis) and the processes that take place during each stage.

OVERVIEW

Not all hematology courses include the material in this chapter. It is a review of basic principles of cellular metabolism and homeostasis, which provide the foundation for understanding many pathologic abnormalities underlying the hematologic disorders in subsequent chapters and thus may be of value to some users. The chapter begins with a review of the basic components and cellular processes of a normal cell and presents the concept of tissue homeostasis. Cellular processes that maintain tissue homeostasis—cell proliferation, cell differentiation, and cell death—are discussed at the functional and molecular level. The chapter concludes with a discussion of what happens when genes controlling cell proliferation, cell differentiation, and/or cell death mutate.

INTRODUCTION

The maintenance of an adequate number of cells to carry out the functions of the organism is referred to as **tissue homeostasis**. It depends on the careful regulation of several cellular processes, including cellular proliferation, cellular differentiation, and cell death (apoptosis). A thorough understanding of cell structural components as well as the processes of cell division and cell death allows us to understand not only the normal (physiologic) regulation of the cells of the blood but also disease processes in which these events become dysregulated (e.g., cancer).

CELL MORPHOLOGY REVIEW

A basic understanding of cell morphology is essential to the study of hematology because many hematologic disorders are accompanied by abnormalities or changes in morphology of cellular or subcellular components and by changes in cell concentrations.

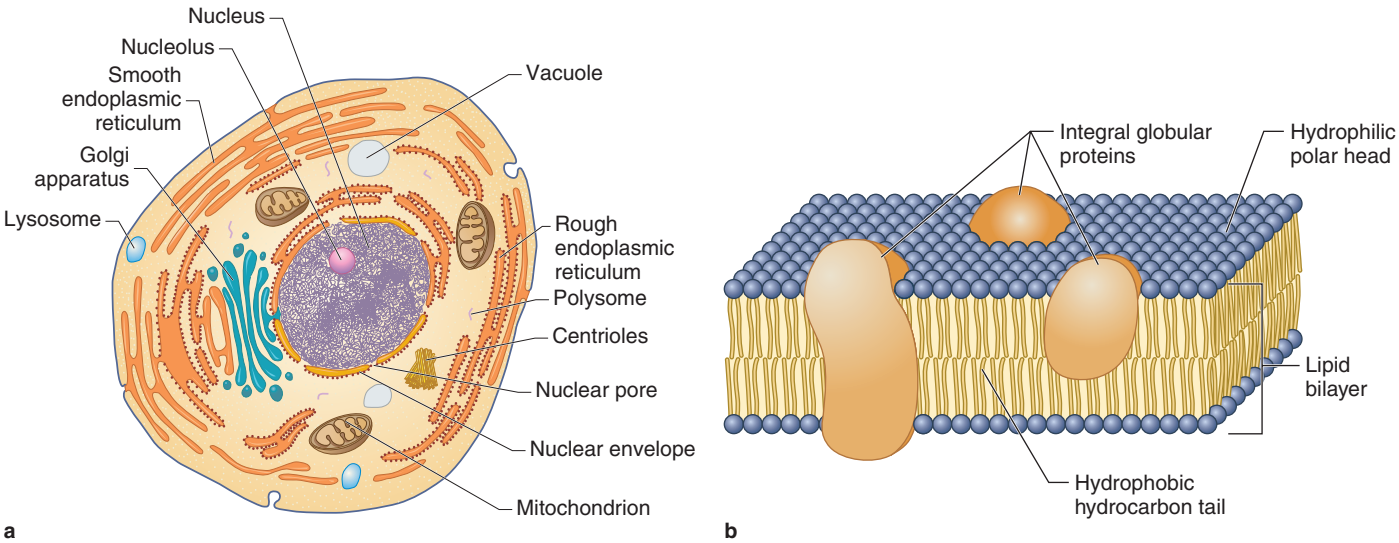
A cell is an intricate, complex structure consisting of a membrane-bound aqueous solution of proteins, carbohydrates, fats, inorganic materials, and nucleic acids. The nucleus, bound by a double layer of membrane, controls and directs the development, function, and division of the cell. The cytoplasm, where most of the cell's metabolic reactions take place, surrounds the nucleus and is bound by the cell membrane. The cytoplasm contains highly ordered organelles, which are membrane-bound components with specific cellular functions (Figure 2-1a ■). The different types of organelles and the quantity of each depend on the function of the cell and its state of maturation.

Cell Membrane

The outer boundary of the cell, the plasma (cell) membrane, is often considered a barrier between the cell and its environment. In fact, it functions to allow the regulated passage of ions, nutrients, and information between the cytoplasm and its extracellular milieu and thus determines the interrelationships of the cell with its surroundings.

The plasma membrane consists of a complex, ordered array of lipids and proteins that serve as the interface between the cell and its environment (Figure 2-1b ■). The plasma membrane is in the form of a phospholipid bilayer punctuated by proteins. The lipids have their polar (hydrophilic) head groups directed toward the outside and inside of the cell and their long-chain (hydrophobic) hydrocarbon tails directed inward. Although the plasma membrane has traditionally been described as a "fluid mosaic" structure,[1] it is in fact highly ordered with asymmetric distribution of both membrane lipids and proteins. The lipid and protein compositions of the outside and inside of the membrane differ from one another in ways that reflect the different functions performed at the membrane's two surfaces.

Four major phospholipids are found in the plasma membrane: phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM) (Web Figure 2-1). Most blood cells have an asymmetric distribution of these phospholipids



■ **FIGURE 2-1** **(a)** Drawing of a cell depicting the various organelles. **(b)** The fluid mosaic membrane model proposed by Singer and Nicholson.

Singer SJ, Nicholson GL. The fluid mosaic model of the structure of cell membranes. *Science*. 1972;175:720–31.

with PE and PS occurring in the inner layer of the lipid bilayer and PC and SM occurring predominantly in the outer layer. The membrane lipids can freely diffuse laterally throughout their own half of the bilayer, or they can flip-flop from one side of the bilayer to the other in response to certain stimuli as occurs in platelets when activated. Membrane lipids including phospholipids, cholesterol, lipoproteins, and lipopolysaccharides contribute to the basic framework of cell membranes and account for the cell's high permeability to lipid-soluble substances. Different mixtures of lipids are found in the membranes of different types of cells.

Although lipids are responsible for the basic structure of the plasma membrane, proteins carry out most of the membrane's specific functions. The proteins of the membrane provide selective permeability and transport of specific substances, structural stability, enzymatic catalysis, and cell-to-cell recognition functions.

The membrane proteins are divided into two general groups: integral (transmembrane) proteins and peripheral proteins. The peripheral proteins are located on either the cytoplasmic or the extracellular half of the lipid bilayer. Some of the integral proteins span the entire lipid bilayer; other integral proteins only partially penetrate the membrane. Some membrane-spanning proteins traverse the membrane once (e.g., erythrocyte glycophorin A) while others cross multiple times (e.g., erythrocyte band 3, the cation transporter). In some cells, such as erythrocytes, peripheral proteins on the cytoplasmic side of the membrane form a lattice network that functions as a cellular cytoskeleton, imparting order on the membrane (Chapter 5).

Carbohydrates linked to membrane lipids (glycolipids) or proteins (glycoproteins) can extend from the outer surface of the membrane. Functions of the carbohydrate moieties include specific binding, cell-to-cell recognition, and cell adhesion. The sugar groups are added to the lipid or protein molecules in the lumen of the Golgi apparatus after synthesis by the endoplasmic reticulum. Many of the glycoprotein transmembrane proteins serve as receptors for extracellular molecules such as growth factors. The binding of the specific ligand to a receptor can result in transduction of a signal to the cell's interior without passage of the extracellular molecule through the membrane (see discussion of cytokine regulation of hematopoiesis, Chapter 4).

Cytoplasm

The cytoplasm, or cytosol, is where the metabolic activities of the cell including protein synthesis, growth, motility, and phagocytosis take place. The structural components, called *organelles*, include the mitochondria, lysosomes, endoplasmic reticulum (ER), Golgi apparatus, ribosomes, granules, microtubules, and microfilaments (Table 2-1 ★). Organelles and other cellular inclusions lie within the cytoplasmic matrix. The composition of the cytoplasm depends on cell lineage and degree of cell maturity. The appearance of cytoplasm in fixed, stained blood cells is important in evaluating the morphology, classifying the cell, and determining the stage of differentiation. Immature or synthetically active blood cells stained with Romanowsky stains (Chapter 37) have very basophilic (blue) cytoplasm due to the large quantity of ribonucleic acid (RNA) they contain.

☑ CHECKPOINT 2-1

What does the phrase *lipid asymmetry* mean when describing cell membranes?

Nucleus

The nucleus contains the genetic material, deoxyribonucleic acid (DNA), responsible for the regulation of all cellular functions. The nuclear material, chromatin, consists of long polymers of nucleotide subunits (DNA) and associated structural proteins (histones) packaged into chromosomes. The total genetic information stored in an organism's chromosomes constitutes its **genome**. The fundamental subunit of chromatin is the nucleosome, a beadlike segment of chromosome composed of about 180 base pairs of DNA wrapped around a histone

★ **TABLE 2-1** Cellular Organelles

Structure	Composition	Function
Ribosomes	RNA + proteins	Assemble amino acids into protein
"Free"	Scattered in the cytoplasm Linked by mRNA-forming polyribosomes	Synthesis of protein destined to remain in cytosol
"Fixed"	Ribosomes bound to outer surface of rough ER	Synthesis of protein destined for export from the cell
ER	Interconnecting membrane-bound tubules and vesicles	Synthesis and transport of lipid and protein
Rough ER	Studded on outer surface with ribosomes	Abundant in cells synthesizing secretory protein; protein transported to Golgi
Smooth ER	Lacks attached ribosomes	Lipid synthesis, detoxification, synthesis of steroid hormones
Golgi apparatus	Stacks of flattened membranes located in juxtanuclear region	Protein from rough ER is sorted, modified (e.g., glycosylated), and packaged; forms lysosomes
Lysosomes	Membrane-bound sac containing catalase, peroxidase, other metabolic enzymes	Destruction of phagocytosed material (extracellular proteins, cells) and cellular organelles (autophagy)
Peroxisome	Membrane-bound sacs containing hydrolytic enzymes	Catabolism of long-chain fatty acids; detoxification of toxic substances
Mitochondria	Double-membrane organelle; inner folds (cristae) house enzymes of aerobic metabolism	Oxidative phosphorylation (ATP production) abundant in metabolically active cells
Cytoskeleton	Microfilaments, intermediate filaments, and microtubules	Gives cell shape, provides strength, and enables movement of cellular structures
Microfilaments	Fine filaments (5–9 nm); polymers of actin	Control shape and surface movement of most cells
Intermediate filaments	Ropelike fibers (~10 nm); composed of a number of fibrous proteins	Provide cells with mechanical strength
Microtubules	Hollow cylinders (~25 mm); composed of protein tubulin	Important in maintaining cell shape and organization of organelles; form spindle apparatus during mitosis
Centrosome	"Cell center"; includes centrioles	Microtubule-organizing center; forms poles of mitotic spindle during anaphase
Centrioles	Two cylindrical structures arranged at right angles to each other; consist of nine groups of three microtubules	Enable movement of chromosomes during cell division; self-replicate prior to cell division

ER = endoplasmic reticulum

protein. The linear array of successive nucleosomes gives chromatin a "beads-on-a-string" appearance in electron micrographs.

The appearance of chromatin varies, presumably depending on activity. The dispersed, lightly stained portions of chromatin (euchromatin) are generally considered to represent unwound or loosely twisted regions of chromatin that are transcriptionally *active*. The condensed, more deeply staining chromatin (heterochromatin) is believed to represent tightly twisted or folded regions of chromatin strands that are transcriptionally *inactive*. In addition to being less tightly associated with the histones, active chromatin characteristically has "unmethylated" promoter regions and highly acetylated histones (see the later section "Epigenetics"). The ratio of euchromatin to heterochromatin depends on cell activity with the younger or more active cells having more euchromatin and a finer chromatin appearance microscopically.

The nuclei of most active cells contain one to four pale staining nucleoli. The nucleolus (singular) consists of RNA and proteins and is believed to be important in RNA synthesis. The nucleolus of very young blood cells is easily seen with brightfield microscopy on stained smears.

A double membrane, the nuclear envelope, surrounds the nuclear contents. The outer membrane (cytoplasmic side) is continuous with the ER and has a polypeptide composition distinct from that of the inner membrane. The gap between the two membranes (~50 nm) is called the *perinuclear space*. The nuclear envelope is interrupted at irregular intervals by openings consisting of nuclear pore complexes (NPCs), which provide a means of communication between nucleus and cytoplasm. NPCs constitute envelope-piercing channels that function as selective gates that allow bidirectional movement of molecules. The nucleus exports newly assembled ribosomal subunits while importing proteins such as transcription factors and DNA repair enzymes.

☑ CHECKPOINT 2-2

Explain the difference between densely staining chromatin and lighter staining chromatin when viewing blood cells under a microscope.

CELLULAR METABOLISM: DNA DUPLICATION, TRANSCRIPTION, TRANSLATION

Genomics is the study of the entire genome of an organism. *Functional genomics* is the study of the actual gene expression "profile" of a particular cell at a particular stage of differentiation or functional activity (i.e., which genes are actively producing mRNA). The morphologic and functional differences between various types of blood cells are governed by which genes are being transcribed/translated into cellular proteins. Microarray or expression array technology can be used to determine the mRNA profile being produced by a cell or tissue of interest, which would reflect which genes are actively being transcribed. The field of

proteomics is the study of the composition, structure, function, and interaction of the proteins being produced by a cell. (Genetic nomenclature has various rules for gene and protein font styles. To differentiate between the gene and its protein, genes are written as italicized capital letters [e.g., *RB*], and the gene's protein product is written with only the first letter capitalized and not italicized. This style is used in this text.)

Genes contain the genetic information of an individual and are found at specific sites on specific chromosomes (gene loci). Most genes are not composed of continuous stretches of nucleotides but are organized into segments called **exons**, which are separated by intervening sequences called **introns**. The exons contain the nucleotide sequences corresponding to the final protein product, while the nucleotide base pairs of the introns do not code for protein. When a gene is transcribed into RNA, the entire sequence of exons and introns is copied as *pre-messenger RNA* (sometimes called *heteronuclear RNA/hnRNA*). Subsequently, the nucleotides corresponding to the intron sequences are spliced out, resulting in the shorter, mature mRNA. Several inherited hematologic diseases, such as some of the thalassemias, result from mutations that derange mRNA splicing (Chapter 14).

Not all of the mature mRNA will be translated into protein. Both the 5′ and the 3′ ends of the mature mRNA encoding the protein to be produced contain **untranslated regions (UTRs)**, which influence the stability of the mRNA and the efficiency of translation to protein. These regions play an important role in regulating many cellular proteins, including those involved in iron metabolism in developing erythrocytes (Chapter 12).

Sometimes large genes with multiple exons can be "read" in a variety of ways, a process described as alternative transcription of the gene. Several different mRNAs and proteins can be produced from a single gene by selective inclusion or exclusion of individual exons from the mature mRNA (i.e., alternative splicing of the pre-mRNA). The human genome is estimated to contain ~30,000 genes; however, alternate transcription and alternate splicing allow for greater genetic complexity than the number of genes would suggest.

Different individuals do not have identical DNA sequences. When a cell replicates its DNA during S phase of the cell cycle (discussed later), the process is not error-free. It has been estimated that ~0.01% of the 6 billion base pairs are copied incorrectly during each S phase.[2] The process of DNA replication is coupled with DNA repair systems to make sure that errors in copying are corrected. If these errors cannot be corrected, the cell may activate its internal apoptotic mechanism (discussed later), resulting in cell death. Errors in DNA replication that cannot be corrected and that subsequently result in activation of apoptosis are believed to be the underlying basis for the large degree of ineffective erythropoiesis in megaloblastic anemias (Chapter 15). In addition to correcting copying errors, DNA repair mechanisms correct other damage to DNA that might have occurred. Failure of these DNA repair mechanisms often contribute to acquired mutations resulting in the development of a malignancy (Chapter 23).

If the miscopied base pair is not corrected, a mutation (or new polymorphism) can occur. Variations in the nucleotide sequence of a gene that can be seen in different individuals are called *alleles*. **Polymorphism** is the term used to describe the presence of multiple alternate copies (alleles) of a gene. Not every alteration in DNA produces an abnormality. For instance, many of the alternate alleles identified for human globin chains do not result in any abnormality of function (Chapter 13). Generally, if the change in DNA sequence does not result in an abnormality of function, the change is called a *polymorphism*. Often the word **mutation** is used only to describe a deleterious change in a gene (e.g., the β^s globin mutation in sickle cell anemia [Chapter 13]).

A region of DNA that differs in only a single DNA nucleotide is called a **single nucleotide polymorphism (SNP)**. SNPs are found at approximately 1 in every 1000 base pairs in the human genome (resulting in ~2.5 million SNPs in the entire genome of a cell). To be considered a true polymorphism, a SNP must occur with a frequency of more than 1% in the general population. If the alteration is known to be the cause of a disease, the nucleotide change is considered to be a mutation rather than a SNP.

Control of Gene Expression

Control of gene expression is a complex process. It must be regulated in both time (e.g., during certain developmental stages) and location (e.g., tissue-specific gene expression). Most genes have a promoter region upstream (5′ side) of the coding region of the gene. **Transcription factors (TFs)** are proteins that bind to the DNA of a target gene's promoter region and regulate expression of that gene. TFs can function to either activate or repress the target gene (some TFs do both, depending on the specific targeted gene). Often TFs are tissue specific, such as GATA-1, a known erythroid-specific TF that regulates expression of glycophorin and globin chains in developing cells of the erythroid lineage.[3]

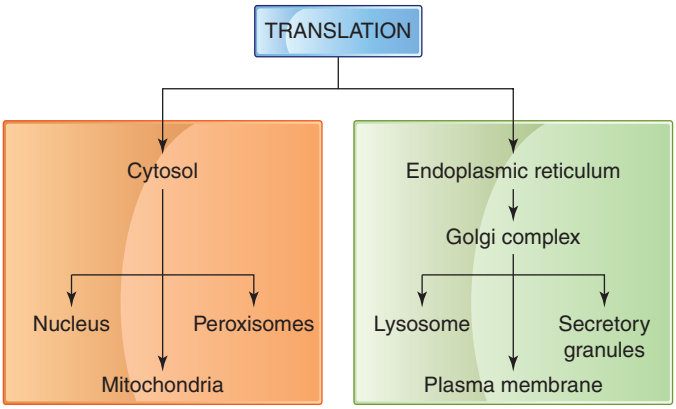
In addition to the basic on/off function of the promoter region, there are additional layers of control of gene expression. Some genes have enhancer elements or silencer elements, which are nucleotide sequences that can amplify or suppress gene expression, respectively.[2] These response elements influence gene expression by binding specific regulatory proteins (transcription activators, transcription repressors).

Many signals that regulate genes come from outside the cell (e.g., cytokine control of hematopoiesis; Chapter 4). The external molecule or ligand (cytokine) binds to its specific receptor on the surface of the cell. The binding of ligand to receptor activates the receptor and initiates a cell-signaling pathway that conveys the activation signal from the receptor to the nucleus. The end result is an interaction with DNA (e.g., TF binding to one or more gene promoter regions) that either activates or represses the target gene(s).

Protein Synthesis and Processing

Synthesis of proteins (polypeptides) occurs on ribosomes. The newly formed polypeptides are transported to their eventual destination through a sorting mechanism within the cytoplasm.[4] If the polypeptide lacks a "signal sequence," translation is completed in the cytosol, and the protein either stays in the cytosol or is incorporated into the nucleus, mitochondria, or peroxisomes. A polypeptide that contains a signal sequence is extruded into the lumen of the ER (which ultimately gives rise to the more distal structures of the secretory apparatus: the Golgi, endosomes, lysosomes, plasma membrane) (Figure 2-2 ■).

Following import into the ER, proteins undergo appropriate folding and possibly **post-translational modifications**. These are modifications in protein structure that occur after the protein is produced by translation on the ribosome. These changes include the addition of nonprotein groups (such as sugars or lipids), modification of existing amino acids (such as the γ-carboxylation of glutamic acid residues on certain coagulation proteins; Chapter 32) or cleavage of the initial



■ **FIGURE 2-2** Proteins are synthesized on ribosomes in the cytoplasm and are targeted for two different pathways. If there is no signal sequence on the polypeptide, translation is completed in the cytosol and the protein is incorporated into the nucleus, mitochondria, peroxisomes, or remains in the cytosol. Polypeptides that have a signal sequence are extruded into the endoplasmic reticulum and are routed to the distal secretory apparatus—the Golgi, lysosomes, endosomes, and plasma membrane.

polypeptide product resulting in a multichain molecule. As the proteins exit the ER, they may be accompanied by molecules that facilitate their transfer from the ER to the Golgi apparatus. A mutation in one of these transfer molecules, ERGIC-53, is the cause of the hemostatic disorder Combined Factor V and VIII deficiency (Chapter 34). During transport through the Golgi, additional processing of the protein can occur.

The primary structure of a protein is defined by its amino acid sequence (see Web Table 2-1 for review of the amino acids and their shorthand notations). A protein emerges from the ribosome in an extended, linear conformation. Subsequently, local regions are folded into specific conformations, the protein's secondary structure, determined by the primary amino acid sequence. The two major secondary protein structures are α-helices and β-pleated sheets. Most proteins are made up of combinations of regions of α-helices and β-pleated sheets connected by regions of less regular structure; these regions are called *loops*. *Molecular chaperones* are cytoplasmic proteins that assist the polypeptide in this folding process. The tertiary structure of a protein refers to its unique three-dimensional shape determined by the folding of secondary structures. Sometimes appropriately folded protein monomers are assembled with other proteins to form multisubunit complexes (also facilitated by chaperones). The quaternary structure of a protein refers to the assembly of independently synthesized polypeptide chains into a multimeric protein (e.g., the $\alpha_2\beta_2$ tetramer, which constitutes hemoglobin A; Chapter 6).

Proteins are often described as being made up of *domains*. Frequently, a domain is encoded in a single exon and represents a region of the polypeptide chain that can fold into a stable tertiary structure. The domains of a protein are often used to designate the location of a particular functional or structural attribute.

☑ CHECKPOINT 2-3

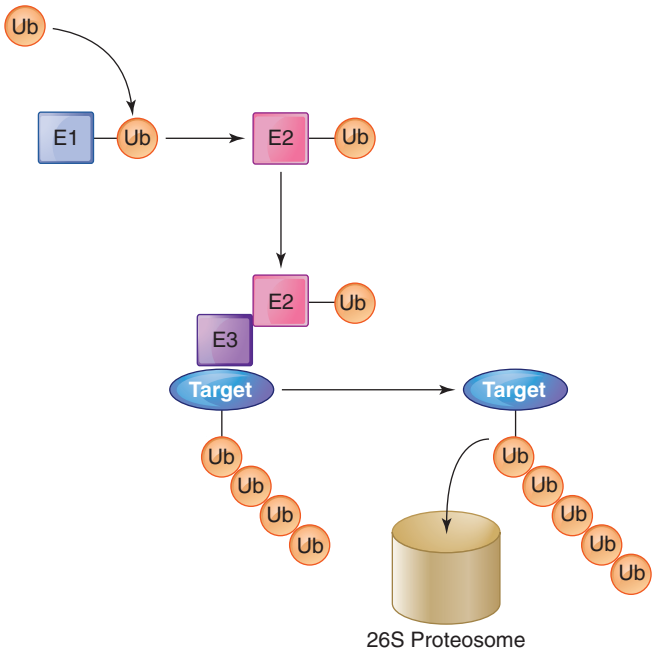
What is the difference between a polymorphism and a mutation?

A mutation that alters a protein's amino acid sequence can result in failure to function. Failure to function can result from either a mutation of a critical functional residue (amino acid) or from the substituted amino acid preventing the protein from folding into its proper three-dimensional structure. Improperly folded proteins are marked for destruction and degraded (via the ubiquitin system).

The Ubiquitin System

Cells contain two major systems for degradation of proteins: the lysosomal system, in which proteolysis occurs within the lysosomes, and the ubiquitin system. The ubiquitin system is a nonlysosomal, proteolytic mechanism in the cytoplasm of most cells that is responsible for disposing of damaged or misfolded proteins.[5] In addition, it regulates numerous cellular processes (e.g., cell-cycle progression, cellular differentiation) by the timed destruction of key regulatory proteins (e.g., cyclins, membrane receptors, transcription factors).

Molecules destined for destruction are tagged with a small (76 amino acid) polypeptide called **ubiquitin** (Figure 2-3 ■). Appropriately labeled molecules are then transferred to an ATP-dependent protease complex (the **proteosome**) for destruction. Generally, proteins bearing a single ubiquitin molecule are marked for endocytosis and degradation in lysosomes. Multi-ubiquitinated proteins are marked for destruction by the proteosome, which is assembled into a cylinder through which proteins are channeled for destruction.



■ **FIGURE 2-3** Ubiquitin-Proteosome system. Ubiquitin-activating enzyme (E1) activates ubiquitin (Ub), which is then transferred to the Ub-conjugating enzyme (E2); Ub ligase (E3) functions in target substrate recognition; it brings together the target and E2-Ub and then catalyzes the transfer of Ub from E2-Ub to the target. Once a target has become multi-ubiquitinated, it is directed to the 26S proteosome for degradation.

TISSUE HOMEOSTASIS: PROLIFERATION, DIFFERENTIATION, AND APOPTOSIS

Tissue homeostasis refers to the maintenance of an adequate number of cells to carry out the organism's functions. In the human body, somatic cells (including blood cells) generally undergo one of three possible fates:

1. Proliferation by mitotic cell division
2. Differentiation and acquisition of specialized functions
3. Death and elimination from the body

Cell proliferation is required for the replacement of cells lost to terminal differentiation, cell death, or cell loss. Differentiation provides a variety of cells, each of which is capable of executing specific and specialized functions. Cell death is also an active process (**apoptosis**) that the cell itself can initiate. Apoptosis is physiologically as important as cell proliferation and differentiation in controlling the overall homeostasis of various tissues. When the regulation of any of these three cellular processes malfunctions or the processes become unbalanced, the consequence may be tissue atrophy, functional insufficiency, or excessive growth/neoplasia (cancer) (Chapters 23–28).

Proliferation: The Cell Cycle

Cell division is required throughout the life of all eukaryotes. Although it has been known for many years that cells have the ability to grow and replicate, the actual mechanisms involved were discovered relatively recently.[6] When a cell is stimulated to divide, it goes through a series of well-defined (biochemical and morphological) stages called the **cell cycle**, which is divided into four phases: G_1 (Gap-1), S (DNA synthesis), G_2 (Gap-2), and M (mitosis) (Figure 2-4 ■).

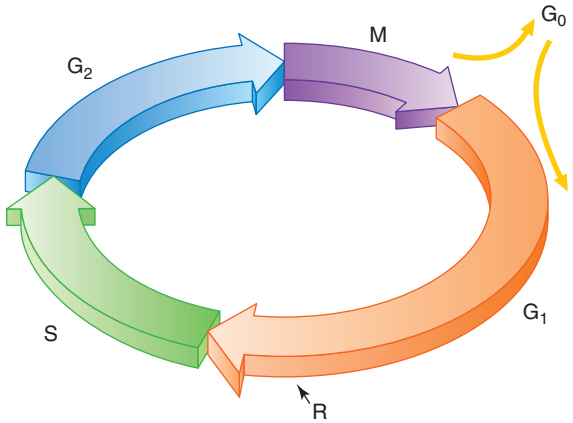
Stages of the Cell Cycle

The physical process of cell division (M phase, or mitosis) includes a series of morphologically recognizable stages (Web Figure 2-2). During mitosis, chromosomes condense (*prophase*) and align on a microtubular spindle (*metaphase*), and sister chromatids segregate to opposite poles of the cell (*anaphase* and *telophase*).

The interval between successive mitoses (known as *interphase*) shows little morphologic variation except that cells increase in volume. During interphase, the cell synthesizes molecules and duplicates its components in preparation for the next mitosis. However, DNA synthesis occurs only within a narrow window of time during interphase. DNA synthesis takes place during *S phase* and is separated from *M phase* (mitosis) by two gap periods: G_1, the time between the end of mitosis and the onset of the next round of DNA replication, and G_2, the time between the completion of S and the onset of mitosis.

Not all of the cells in the body are actively dividing (i.e., actively engaged in the cell cycle). Cells can exit the cell cycle at G_1 and enter a nonproliferative phase called G_0, or **quiescence** (Figure 2-4). To proliferate, a quiescent cell must re-enter the cell cycle. In response to specific mitogenic stimuli or growth factors, quiescent cells can exit G_0 and re-enter the cell cycle at the level of early G_1. In unicellular organisms such as bacteria, cell division depends only on an adequate supply of nutrients. In mammalian cells, all cell division cycles are initiated by specific growth factors or mitogens that drive the cell from G_0 to G_1 ($G_0 \rightarrow G_1$). Some cells, such as terminally differentiated neutrophils, have irreversibly exited the cell cycle during differentiation and are locked in G_0. Other cells, such as hematopoietic stem cells or antigen-specific memory lymphocytes, primarily reside in G_0 but can be induced to return to G_1 and begin cycling with appropriate cytokine or antigen stimulation.

G_1 is characterized by a period of cell growth and synthesis of components necessary for cellular replication. If conditions are unsuitable for proliferation (insufficient nutrients or mitogens) cells will arrest in G_1. As cells transit through the G_1 phase of the cell cycle, they pass through what is called the **restriction point (R)** in late G_1. R defines a point in the cell cycle after which the cell no longer depends on extracellular signals but is committed to completing that cell cycle *independent* of further mitogen stimulation (i.e., cell-cycle completion becomes autonomous).[6] Cells then transit across the G_1/S boundary into S phase where DNA synthesis occurs, followed by the G_2 phase, and finally mitosis where nuclear division (*karyokinesis*) and cytoplasmic separation (*cytokinesis*) occur.



■ **FIGURE 2-4** The four phases of the cell cycle: G_1, S, G_2, and M. G_0 represents the state of quiescence when a cell is withdrawn from the cell cycle. R represents the restriction point—the point in the cell cycle after which the cell no longer depends on extracellular signals but can complete the cycle in the absence of mitogenic stimuli.

Molecular Regulation of the Cell Cycle

The fundamental task of the cell cycle is to faithfully replicate DNA once during the S phase and to distribute identical copies of each chromosome to each daughter cell during M phase. Progression through the cell cycle normally ensures that this takes place, so cells do not initiate mitosis before DNA duplication is completed, do not attempt to segregate sister chromatids until all chromosome pairs are aligned on the mitotic spindle at metaphase, and do not reduplicate their chromosomes (reinitiate S phase) before the paired chromatids have been separated at the previous mitosis. Cells must ensure that chromosome duplication and segregation occur in the correct order (i.e., S → M → S → M). They must also ensure that the next event in the cycle begins *only* when the previous events have been successfully completed (i.e., chromosome duplication is complete before the chromosomes are segregated into the two daughter cells). Entry into and exit from each cell-cycle phase are tightly regulated. Failure to regulate this process results in aneuploidy (abnormal chromosome number)

seen in many malignancies. Research over the past 25 years has begun to reveal how cells guarantee the orderliness of this process.[7]

Cyclins and Cyclin-Dependent Kinases

Enzymatic activities of specific kinases (phosphorylating enzymes) regulate the transition between the various phases of the cell cycle. These kinase proteins (**Cdks**, or cyclin-dependent kinases) phosphorylate target molecules important for cell cycle control. To be active, the kinase (Cdk) must be complexed with a regulatory protein named **cyclin** (hence the name, **c**yclin-**d**ependent **k**inase). The concentration of the different cyclin proteins that regulate the cell cycle rises and falls at specific times during the cell cycle (hence, they are *cycling* proteins). Sequential activation of unique complexes with differing cyclin and Cdk components drive the cell from one cell-cycle stage to the next as summarized in Table 2-2 ★. Each complex in turn phosphorylates key substrates and facilitates or regulates the movement of the cell through the cycle (Figure 2-5a ■). Cdk inhibitors that function to inhibit the kinase activity by binding to the Cdks or the Cdk/cyclin complexes also exist.

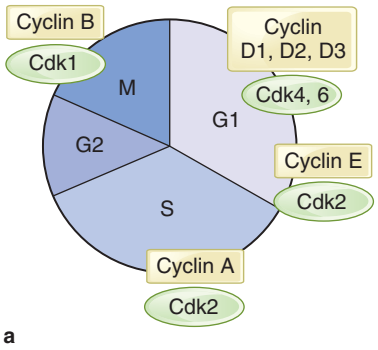
A mammalian cell must receive external signals (growth factors and/or hormones) that trigger the cell to proliferate.[8] These external signals result in an increase of one (or more) of the D cyclins (of which there are three: D1, D2, D3). Cyclin D complexes with Cdk4 or Cdk6 and phosphorylates target molecules required for $G_1 \rightarrow S$ progression. The D cyclins are unique in that they are synthesized in response to growth factor stimulation and remain active in the cell as long as the mitotic stimulus is present. During mid to late G_1, levels of cyclin E increase and bind with Cdk2. The cyclin E/Cdk2 complex is required for the $G_1 \rightarrow S$ transition. Once the cell enters S phase, cyclin E degrades rapidly, and cyclin A takes over the activation of Cdk2. Cyclin A/Cdk2 is required for the onset of DNA synthesis and progression through S phase. Toward the end of S phase, cyclin A starts to activate another kinase, Cdk1, which signals the completion of S phase and the onset of G_2. Cyclin B (which begins to increase during S and G_2) takes over from cyclin A as the activating partner of Cdk1, and cyclin B/Cdk1 controls the onset, sequence of events, and the completion of mitosis. Cyclin B must be destroyed for the cell to exit mitosis and for cytokinesis to take place (Figure 2-5b ■).

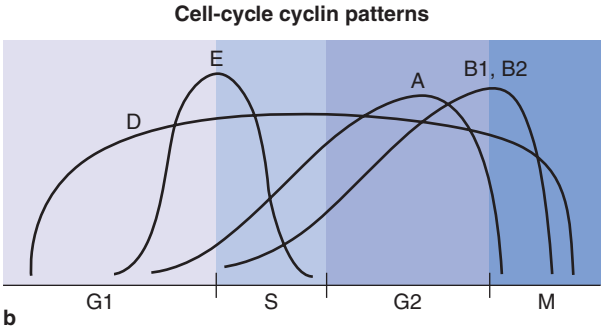
Regulation of Cell-Cycle Kinase Activity

Control of cell-cycle kinase activity is somewhat unique in that protein levels of the enzyme (kinase) subunit remain constant throughout the cell cycle and do not require activation from a proenzyme precursor form. The cell cycle is regulated by altering the availability of the regulatory cofactor (the cyclins) through periodic (and cell-cycle phase-specific) synthesis and degradation of the appropriate cyclin via the ubiquitin system[9] (Figure 2-5b). The periodic accumulation

★ **TABLE 2-2** Cell-Cycle Regulatory Proteins

Cell-Cycle Phase	Cyclin	Partner Cdk
G_1	D1, D2, D3	Cdk4, Cdk6
G_1/S	E	Cdk2
S	A	Cdk2
G_2/M	A	Cdk1
M	B	Cdk1



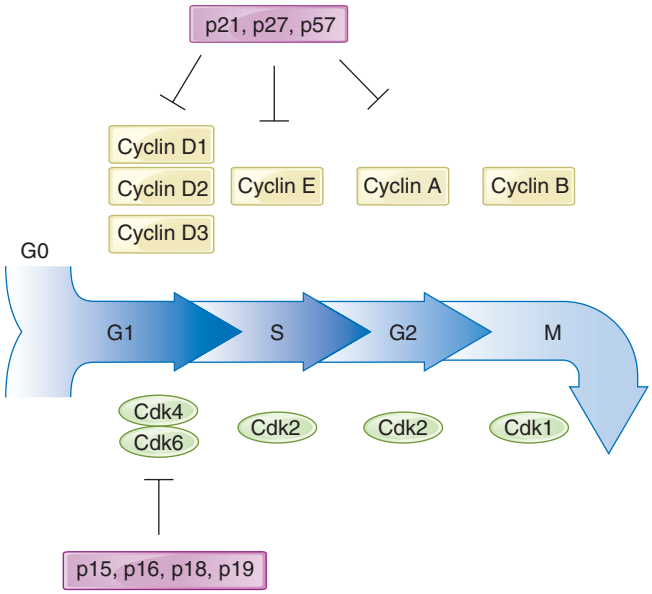


■ **FIGURE 2-5** **(a)** The phases of the cell cycle with the major regulatory cyclin/Cdk complexes depicted for each. **(b)** The levels of the various cyclin proteins during the cell cycle. The cyclins rise and fall in a periodic fashion, thus controlling the cyclin-dependent kinases and their activities.

of different cyclins determines the sequential rise and fall of kinase activities, which in turn regulate the events of cell-cycle progression.

Multiple mechanisms regulate cell-cycle kinase activity. In addition to requiring the appropriate cyclin partner, the kinase subunit (Cdk) must be phosphorylated and/or dephosphorylated at specific amino acid residues in order to have a fully active cyclin/Cdk complex[10] (Web Figure 2-3). The kinase responsible for this activating phosphorylation is CAK (**C**dk-**a**ctivating **k**inase) and is itself a Cdk (Cdk7 complexed with cyclin H). CAK is responsible for activating phosphorylation of *all* the kinases important for mammalian cell-cycle control. On the other hand, phosphorylation of certain amino acids suppresses kinase activity, and these inhibitory phosphates must be removed (by the phosphatase Cdc25) to have a fully active cyclin/Cdk complex (Web Figure 2-3).

The final level of regulation involves two groups of proteins that function as inhibitors of Cdks and cyclin/Cdk complexes[11] (Figure 2-6 ■). The first Cdk inhibitor identified was p21; other Cdk inhibitors with structural and functional similarities to p21 include p27 and p57. (This nomenclature indicates that they are **p**roteins of the indicated molecular mass in kilodaltons [e.g., p21 is a protein of molecular weight of 21,000]). These three inhibitors are considered "universal" because they bind multiple cyclin/Cdk complexes of various phases of the cell cycle (cyclin D/Cdk4/6, cyclin E/Cdk2, and cyclin A/Cdk2). The second group of inhibitors is a family of structurally related proteins that include p15, p16, p18, and p19. These inhibitors are more restricted in their inhibitory activity, inhibit only Cdk4 and Cdk6, and induce cell-cycle arrest in G_1.



■ **FIGURE 2-6** Cdk inhibitors. There are two families of cyclin-dependent kinase inhibitors. The first group, including p15, p16, p18, and p19, inhibits only D-type cyclin/Cdk4 or Cdk6 complexes. The second group of inhibitors, including p21, p27, and p57, possesses a wider spectrum of inhibitory activity, inhibiting the G_1 as well as S phase cyclin/Cdk complexes (cyclin D/Cdk4/6, cyclin E/Cdk2, and cyclin A/Cdk2). ⊥ indicates inhibition of the pathway.

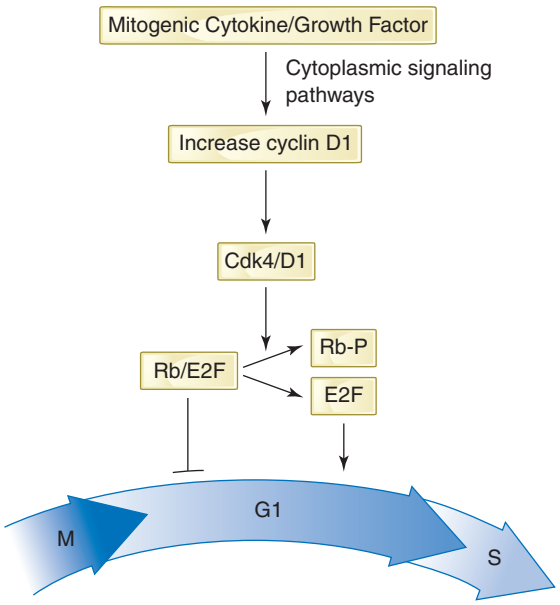
Cell-Cycle Checkpoints

Cell proliferation and differentiation depend on the accurate duplication and transfer of genetic information, which requires the precise ordering of cell-cycle events. Cells achieve this coordination by using **cell-cycle checkpoints** to monitor events at critical points in the cycle and, if necessary, halt the cycle's progression.[12–14] The main functions of checkpoints are to detect malfunctions within the system and to assess whether certain events are properly completed before the cell is allowed to proceed to the next phase of the cycle. When problems are detected, checkpoint mechanisms interrupt cell cycling to allow correction of the problem or elimination of the defective cell.

Four major cell-cycle checkpoints have been described. The *G_1 checkpoint* checks for DNA damage and prevents progression into S phase if the genomic DNA is damaged. The *S-phase checkpoint* monitors the accuracy of DNA replication. The *G_2/M checkpoint* also monitors the accuracy of DNA replication during S phase and checks for damaged or unreplicated DNA; it can block mitosis if any is found. The *metaphase checkpoint* (also called the *mitotic-spindle checkpoint*) functions to ensure that all chromosomes are properly aligned on the spindle apparatus prior to initiating chromosomal separation and segregation at anaphase. If defects are detected at any of these checkpoints, the cell cycle is stopped and repair pathways are initiated, or if the damage is severe and/or irreparable, apoptosis can be triggered (see the section "Apoptosis"). Mechanisms that detect damaged DNA include two important proteins, ATM (**a**taxia-**t**elangiectasia **m**utated) and ATR (**AT** and **R**AD-3-related) kinases.[15] Both are activated by damaged DNA and in turn phosphorylate effectors of the checkpoint response including the proteins Chk1 and Chk2. These proteins in turn activate p53 and/or inhibit Cdc25, which inhibit the cyclin/Cdk complexes or trigger apoptosis.

Two proteins critical for regulation of the cell cycle are p53 and Rb. Rb is the protein product of a gene (*RB*) that predisposes individuals to retinoblastomas and other tumors when only one functional copy of the gene is present. Rb is present throughout the cell cycle, although its phosphorylation state changes markedly at different phases (Figure 2-7 ■).[16, 17] In its *hypo*phosphorylated (active) state, Rb inhibits cell-cycle progression (proliferation) by binding transcription factors (the E2F proteins) that are required for the transcription of genes needed for cell proliferation, thus rendering them nonfunctional. When growth factors induce activation of cyclin D/Cdk4/6, the Rb protein is phosphorylated by this kinase activity. As cells progress through G_1, *hyper*phosphorylation of Rb by cyclin D/Cdk4/6 kinase results in the inactivation of Rb, the release of the active E2F transcription factors, and the activation and expression of genes required for cell-cycle progression. Cyclin E/Cdk2 and cyclin A/Cdk2 subsequently maintain Rb hyperphosphorylation through the cell cycle. *RB* functions as a tumor suppressor gene. Cells that lack functional Rb protein have deregulation of cell-cycle genes and cell proliferation, sometimes resulting in malignancy.

The protein p53 is not required for normal cell function (i.e., it is not *required* for cell-cycle progression). However, it serves an important function as a molecular policeman that monitors the genome's integrity.[18]



■ **FIGURE 2-7** The role of the retinoblastoma susceptibility gene product (Rb) in regulation of the cell cycle. Stimulation of a cell with mitogens or growth factors induces synthesis of the D-type cyclins. Activation of G_1 phase kinase activity (cyclin D/Cdk4/6) phosphorylates a number of intracellular substrates including the Rb protein. In the hypophosphorylated (active) state, Rb binds and sequesters transcription factors known as E2F, rendering them inactive. When cyclin D/Cdk4 or Cdk6 phosphorylates Rb, it releases the E2F transcription factors, which then move to the nucleus, and initiate transcription of genes required for cell-cycle progression (including the genes for cyclin E and cyclin A). ↓ indicates stimulation of the pathway; ⊥ indicates inhibition of the pathway.

p53 is a transcription factor that can both activate and inhibit gene expression, depending on the target gene. It is induced when DNA damage is detected and puts the brakes on cell growth and division. This allows time for DNA repair or can trigger apoptosis if repair is not possible.

Elevated levels of p53 result in upregulation of the Cdk inhibitor p21 and inhibition of the Cdc25 phosphatase (blocking kinase function), induction of pro-apoptotic Bax, and inhibition of anti-apoptotic Bcl-2 (see the section "Apoptosis"). p53 is an important component of both the G_1 and the G_2/M checkpoints. Like *RB, p53* functions as a tumor suppressor gene and is the most commonly mutated gene in human malignancies.

☑ CHECKPOINT 2-4

A cell undergoing mitosis fails to attach one of its duplicated chromosomes to the microtubules of the spindle apparatus during metaphase. The cell's metaphase checkpoint malfunctions and does not detect the error. What is the effect (if any) on the daughter cells produced?

Differentiation

Differentiation is the process that generates the diverse cell populations found throughout the body. All cells in the human body contain the exact same genetic information. The appearance of specific characteristics in various cell populations is dictated by the specific genes that are actively being transcribed into mRNA and the translation of that genetic information into functional proteins. Regulation of gene expression is controlled at various levels.

The transcription of genes is regulated by binding transcription factors to the promoter regions of the genes that encode for proteins specific for the given type of cell, resulting in tissue-specific mRNAs. As differentiation progresses within a given tissue or cell lineage, different genes will be sequentially activated and inactivated, resulting in a changing landscape of mRNAs and proteins that drive the differentiation process. Two additional cell systems, epigenetics and mRNA interference, are important in the regulation of this process.

Epigenetics

Epigenetics (meaning literally "on top of genetics") refers to stable changes in gene function that are passed from one cell to its progeny. Epigenetic changes play an important role in normal development and differentiation and are associated with "silencing" genes and chromatin condensation into heterochromatin.[19]

One of the most common epigenetic changes found in the human genome involves the methylation of certain cytosine nucleotides (C^M) within genes and/or their promoter regions.[20] Cytosine nucleotides found adjacent to a guanine nucleotide, the so-called CpG dinucleotide, are particularly susceptible to methylation.

$$CGATCGATCGAT \rightarrow C^MGATC^MGATC^MGAT$$

These methylations or epigenetic changes become incorporated into the genetic/epigenetic regulatory mechanisms of the cell, are conserved during subsequent cell divisions, and play a significant role in cellular differentiation pathways. The methylation of CpG dinucleotides is a potentially reversible process, and approximately 70–75% of CpG dinucleotides in the human genome are methylated. In addition, CpG dinucleotides are often clustered in *CpG islands*, many of which are in and around the promoter regions of genes. The unmethylated state of a gene's promoter region indicates a *transcription-ready status* and is seen in genes actively being transcribed into mRNA. Typically, methylation of the promoter regions is associated with gene silencing and is part of the normal terminal differentiation process seen in many diverse tissue types.

Extensive information also can be encoded in the protein component of the chromatin in what is called the *histone code*. Modifications of the histone proteins include lysine acetylation, serine phosphorylation, and lysine and arginine methylation.[21] These modifications can also be passed from one cell generation to the next during cell division and play an important role in the complex system responsible for regulating euchromatin to heterochromatin transitions. Hypoacetylated histones bind tightly to the phosphate backbone of DNA and help maintain chromatin in an inactive, silent state. Acetylated histones maintain a more relaxed chromatin structure and allow gene transcription to occur.[22] Both DNA methylation and histone hypoacetylation promote chromatin condensation and gene silencing.

As cells go through a particular differentiation program, the DNA methylation patterns and histone acetylation/deacetylation patterns change as successive genes are activated and deactivated. It is possible to map the DNA methylation patterns within a cell by using a method called *microarray analysis*, and this can be useful in evaluating a variety of diseases, including cancer.

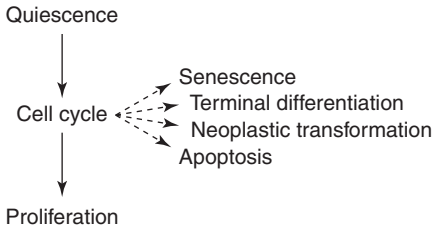
Translational Regulation

Genomic expression in the context of which proteins are produced within a cell is controlled not only at the level of gene transcription (production of mRNA) but also by post-transcriptional events that affect mRNA stability. Interfering with the function of mRNA (RNA interference, RNAi) can also modify the function of genes.[2]

Two forms of RNA are involved in regulating translation of mRNA, micro-RNA (miRNA) and small interfering RNA (siRNA). Both types of molecules function in RNAi pathways and block protein expression by inhibiting translation or inducing degradation of their respective target mRNA (and thus "gene silencing"). The exact mechanisms of RNAi function in normal cellular biology, and the contribution of RNAi to various pathologic states are areas of active ongoing research.

Apoptosis

Cells stimulated to enter the cell cycle can experience outcomes other than proliferation (Figure 2-8 ■). Cells can undergo senescence in which they are permanently growth arrested and no longer respond to mitogenic stimuli. Cells entering the cell cycle can also become



■ **FIGURE 2-8** Alternative fates for a cell induced to enter the cell cycle.

★ **TABLE 2-3** Cardinal Features of Apoptosis and Necrosis

Feature	Necrosis	Apoptosis
Stimuli	Toxins, severe hypoxia, massive insult, conditions of ATP depletion	Physiologic and pathologic conditions without ATP depletion
Energy requirement	None	ATP dependent
Histology	Cellular swelling; disruption of organelles; death of patches of tissue	Cellular shrinkage; chromatin condensation; fragmentation into apoptotic bodies; death of single isolated cells
DNA breakdown pattern	Randomly sized fragments	Ladder of fragments in internucleosomal multiples of 185 base pairs
Plasma membrane	Lysed	Intact, blebbed with molecular alterations (loss of phospholipid asymmetry)
Phagocytosis of dead cells	Immigrant phagocytes	Neighboring cells
Tissue reaction	Inflammation	No inflammation

terminally differentiated (committed) into specialized cell types. Uncontrolled cell cycling is a characteristic feature of malignant cells. Finally, cells can exit at any phase of the cell cycle by undergoing programmed cell death (apoptosis).

Cells can die by either **necrosis** or **apoptosis**. The criteria for determining whether a cell is undergoing apoptosis or necrosis originally relied on distinct morphologic changes in the appearance of the cell[23] (Table 2-3 ★). Necrotic death is induced by lethal chemical, biological, or physical events (extracellular assault). Such a death is analogous to "cell murder." In contrast, apoptosis, or "programmed cell death," is a self-induced death program regulated by the cell itself ("cell suicide").

Apoptosis plays an essential role in the development and homeostasis of all multicellular organisms.[24] Apoptosis helps maintain a constant organ size in tissues that undergo continuous renewal, balancing cell proliferation and cell death. It also occurs at defined times and locations during development.

In adults, apoptosis is also important in tissue homeostasis; homeostasis generally balances generation of new cells with the loss of terminally differentiated cells. Apoptosis is responsible for the elimination of excess cells (such as expanded clones of T or B lymphocytes following immune stimulation, or excess neutrophils following a bacterial challenge). As a defense mechanism, apoptosis is used to remove unwanted and potentially dangerous cells such as self-reactive lymphocytes, cells infected by viruses, and tumor cells. Diverse forms of cellular damage can trigger apoptotic death including DNA damage or errors of DNA replication, which prevent the cell with abnormal DNA from proliferating. Similarly, intracellular protein aggregates or misfolded proteins can stimulate apoptosis (e.g., the ineffective erythropoiesis and intramedullary apoptotic death of erythroblasts in β-thalassemia major triggered by aggregates of α globin chains [Chapter 14]). In addition to the beneficial effects of programmed cell death, the inappropriate activation of apoptosis can cause or contribute to a variety of diseases[25,26] (Table 2-4 ★).

Apoptosis is initiated by three major types of stimuli (Table 2-5 ★):

1. Deprivation of survival factors (growth factor withdrawal or loss of attachment to extracellular matrix)
2. Signals by "death" cytokines through apoptotic "death" receptors (tumor necrosis factor [TNF], Fas ligand)
3. Cell-damaging stress

Conversely, apoptosis is inhibited by growth-promoting cytokines and interaction with appropriate extracellular environmental stimuli. The disruption of cell physiology as a result of viral infections can cause an infected cell to undergo apoptosis. This suicide of

★ **TABLE 2-4** Diseases Associated with Increased and Decreased Apoptosis

Increased Apoptosis	Decreased Apoptosis
AIDS	Cancer
Neurodegenerative disorders	• Follicular lymphomas
• Alzheimer's disease	• Other leukemias/lymphomas
• Parkinson's disease	• Carcinomas with *p53* mutations
• Amyotrophic lateral sclerosis	• Hormone-dependent tumors (breast, prostate, ovarian)
• Retinitis pigmentosa	Autoimmune disorders
Myelodysplastic syndromes	• Systemic lupus erythematosus
Aplastic anemia	• Other autoimmune diseases
Ischemic injury	Viral infections
• Myocardial infarction	• Herpes viruses
• Stroke	• Poxviruses
• Reperfusion injury	• Adenoviruses
Toxin-induced liver disease	

★ **TABLE 2-5** Inhibitors and Initiators/Inducers of Apoptosis

Inhibitors	Initiators/Inducers
Presence of survival factors • Growth factors • Extracellular matrix • Interleukins • Estrogens, androgens	Deprivation of survival factors • Growth factor withdrawal • Loss of matrix attachment
Viral products that block apoptosis • Cowpox virus CrmA • Epstein Barr virus BHRF-1	Death cytokines • TNF • Fas ligand
Pharmacologic inhibitors	Cell-damaging stress • Bacterial toxins • Viral infections • Oxidants • Glucocorticoids • Cytotoxic drugs • Radiation therapy
Oncogene and tumor suppressor gene products (Bcl-2, Bcl-X_L, Mcl-1, Rb, c-Abl)	Oncogene and tumor suppressor gene products (c-myc, p53, Bax, Bad, BCL-X_S, c-Fos, c-Jun)

an infected cell can be viewed as a cellular defense mechanism to prevent viral propagation. To circumvent these host defenses, a number of viruses have developed mechanisms to disrupt the normal regulation of apoptosis within the infected cell. Finally, a number of oncogenes and tumor suppressor genes that can either stimulate or inhibit apoptosis have been described (Chapter 23).

Necrosis versus Apoptosis

When a cell is damaged, the plasma membrane often loses its ability to regulate cation fluxes, resulting in the accumulation of Na^+, Ca^{++}, and water (Table 2-3). Consequently, the necrotic cell exhibits a swollen morphology. The organelles also accumulate cations and water, swell, and ultimately lyse. The rupture of the cytoplasmic membrane and organelles releases cytoplasmic components (including proteases and lysozymes) into the surrounding tissue, triggering an inflammatory response. In contrast, apoptosis is characterized by cellular shrinking rather than swelling, with condensation of both the cytoplasm and the nucleus. Apoptotic cells do not lyse, but portions of the cells pinch off as apoptotic bodies that are phagocytized by neighboring cells or macrophages. Thus, apoptosis is a very efficient process by which the body can remove a population of cells at a given time or in response to a given stimulus without the activation of an inflammatory response.

Necrosis is a passive event induced by the external injurious agent and generally leads to the destruction of a large group of cells in the same area. In contrast, apoptosis is an energy-dependent process orchestrated by the cell itself and generally affects only individual cells. In addition, a particular type of DNA fragmentation characterizes apoptosis. DNA in an apoptotic cell is enzymatically cleaved by a specific endonuclease into oligonucleotides whose sizes are multiples of ~ 185 base pairs (corresponding to nucleosomal fragments). When electrophoresed on agarose gel, these nucleotide fragments make a discrete "ladder pattern" that is considered the hallmark of apoptosis. This is in contrast to the "smudge" pattern seen in cells undergoing necrosis, which indicates the presence of randomly degraded DNA.

Molecular Regulation of Apoptosis

Apoptosis is a closely regulated physiologic process that involves a group of proteins called *caspases* and the *Bcl-2 family of proteins*.

Role of Caspases and the Initiation of Apoptosis

The cellular events responsible for apoptotic cell death are directed by **caspases**,[27,28] a family of **c**ysteine proteases that cleave after **asp**artic acid amino acids in a peptide substrate and are responsible for the orderly dismantling of the cell undergoing apoptosis.

At least 14 caspase enzymes (caspase 1–14) have been identified in humans, although not all play a significant role in apoptosis. Those that are involved in apoptosis form the effector arm of the apoptotic machinery that, once activated, carries out the proteolysis necessary for apoptosis to occur. Caspases are found in healthy cells as zymogens (inactive form) and express their protease activity following either proteolytic cleavage or autocatalytic activation at high concentrations. A hierarchical relationship similar to that described for the blood coagulation proteins exists among the apoptotic caspases. Early acting, initiator caspases (e.g., caspase-2, -8, -9, -10) are recruited in response to apoptotic stimuli and are activated. They then initiate the apoptotic cascade by proteolytically activating downstream effector caspases (e.g., caspase-3, -6, -7), which in turn orchestrate the cell's death[28,29] (Figure 2-9 ■). Activation of caspases in apoptosis does not lead to indiscriminate proteolytic degradation but to specific cleavage of key substrates including proteins involved in cell structure, cell-cycle regulation, transcription, translation, DNA repair, and RNA splicing. One key substrate activated by caspases is an endonuclease (CAD/**c**aspase-**a**ctivated **D**NAse) that is responsible for the characteristic DNA fragmentation (Web Table 2-2).

Two major cell death pathways (Web Figure 2-4) are initiated by a variety of events. Similar to the coagulation cascade (Chapter 32), there is an "extrinsic pathway" and an "intrinsic pathway." The extrinsic pathway is triggered by extracellular signals ("death cytokines") and transmitted through "death receptors" on the surface of the cell. The intrinsic pathway is a mitochondria-dependent pathway initiated by intracellular signals in response to stress, exposure to cytotoxic agents, DNA damage, or radiation.

At least eight death receptors have been described in mammalian cells to date.[30–32] The two best-known death cytokines and death receptors (DR) are (1) tumor necrosis factor (TNF) and the TNF receptor and (2) Fas Ligand and CD95 (Fas receptor). DRs do not bind initiator caspases directly but interact through adapter molecules containing "docking sites" or domains for each protein (Web Figure 2-5). Once the

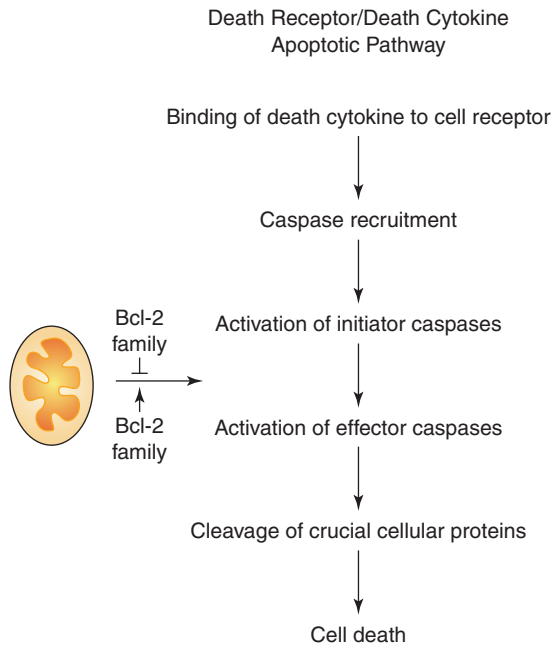


FIGURE 2-9 The apoptotic pathway triggered by death cytokine binding to death receptors. Activation of a death receptor by binding of death cytokine results in the recruitment of specific adapter proteins and activation of initiator caspases. Activated initiator caspases can then proceed to activate downstream effector caspases that mediate the cleavage of various cellular proteins during apoptosis. The contribution of the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins in determining whether activation of initiator caspases will proceed through to activation of effector caspases is depicted. ↓ indicates stimulation of the pathway; ⊥ indicates inhibition of the pathway.

death cytokine, death receptor, adapter molecules, and initiator caspases are assembled in a complex called DISC (**D**eath-**I**nducing **S**ignaling **C**omplex), the caspase is activated by the process of autocatalysis.

The sequence of events triggering apoptosis via the intrinsic pathway is less understood. It involves the assembly of a second caspase-activating complex called the *apoptosome* (Web Figure 2-5). DNA damage, triggering cell-cycle checkpoints, or loss of survival factors increase expression of pro-apoptotic Bcl proteins (discussed in the section "Role of Bcl-2 Proteins") and trigger mitochondrial release of cytochrome-c that serves as a cofactor for caspase activation. Cytochrome-c assembles with a different adapter protein and initiator caspase, again triggering autocatalysis. The activated initiator caspases from both pathways converge on the proteolytic activation of the effector caspase, caspase-3, and trigger apoptosis.

Role of Bcl-2 Proteins

The Bcl-2 family of proteins includes both pro-apoptotic and anti-apoptotic members and constitutes a critical intracellular checkpoint regulating apoptosis.[33,34] The founding member, Bcl-2, was a protein originally cloned from B-cell lymphomas with the characteristic t(14;18) chromosomal translocation (Chapter 28). Since that initial discovery, several additional proteins related to Bcl-2 have been identified, some of which promote and others oppose apoptosis. At present, there are at least 6 known apoptosis-inhibitory proteins (survival factors) including the originally described Bcl-2 and at least 14 pro-apoptotic family members.[32] The Bcl-2 family of proteins is localized at or near the mitochondrial membranes and constitutes a critical intracellular checkpoint of apoptosis. Bcl protein interactions determine whether early activation of initiator caspases proceeds to full activation of effector caspases (see Figure 2-9).[35,36]

The relative levels of anti-apoptotic and pro-apoptotic Bcl-2 family members constitute a *rheostat* for apoptosis. This rheostat is regulated by different associations between prosurvival and prodeath proteins, all of which share similar structural regions that allow them to form dimers or higher oligomers (either homo- or hetero-oligomers). Bax, the first pro-apoptotic member discovered, can associate with itself (Figure 2-10 ■); Bax:Bax homo-oligomers promote apoptosis. They induce permeabilization of the mitochondrial membrane and release of proteins, including cytochrome-c, and activation of the caspase cascade. When Bcl-2 is increased, Bax:Bcl-2 hetero-oligomers form and repress apoptosis. Actually, it is the overall ratio of various death agonists (Bax and related proteins) to death antagonists (Bcl-2 and related proteins) and their interactions with each other that determine the susceptibility of a cell to a death stimulus (Figure 2-11 ■).

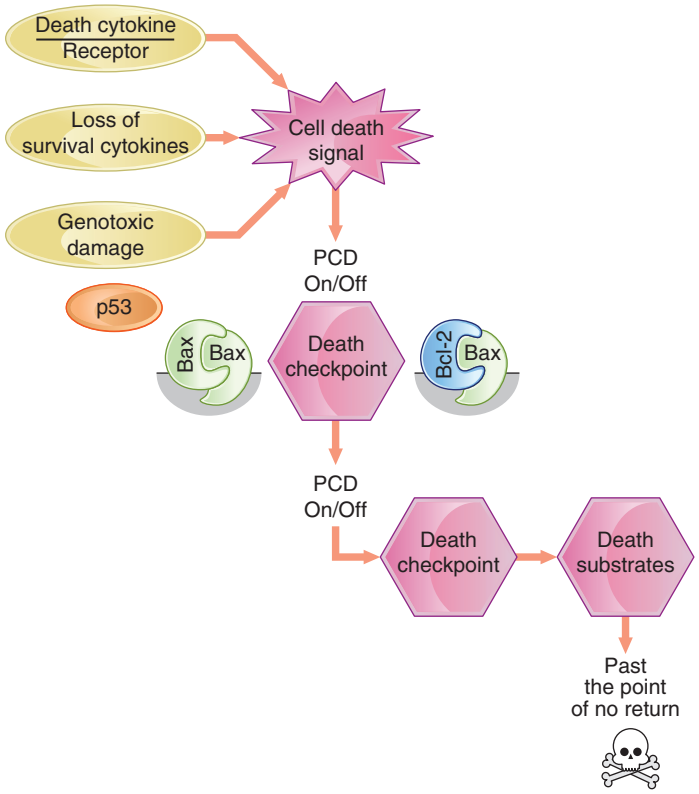
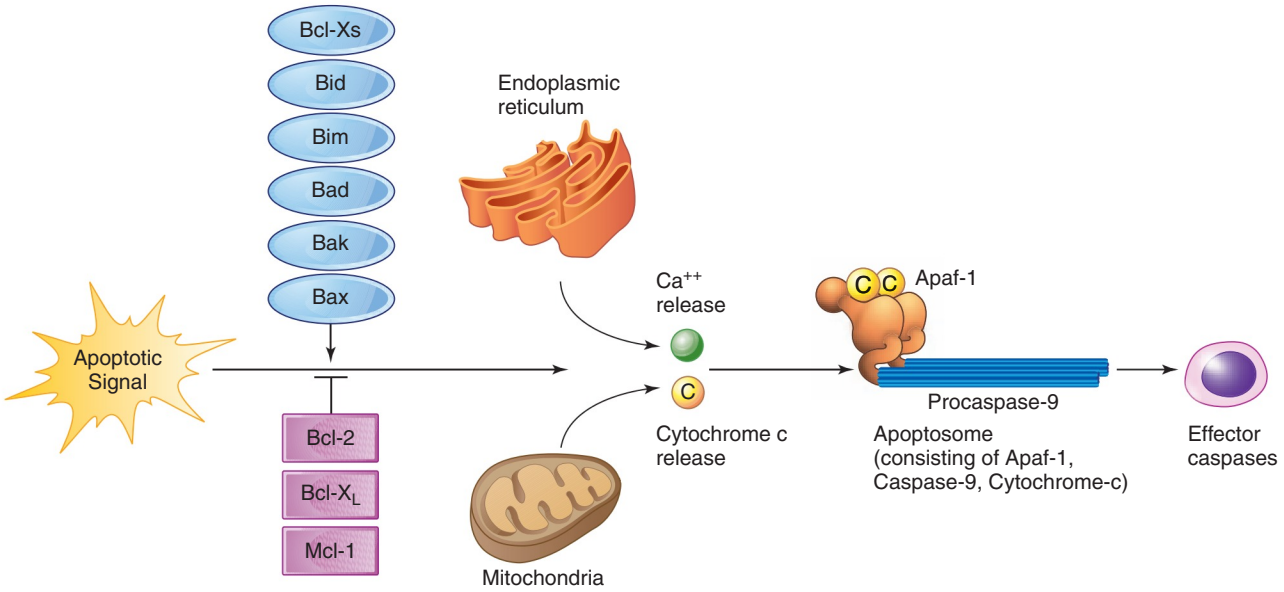


FIGURE 2-10 Model of cell death checkpoints. Following delivery of a cell death signal (genotoxic damage, loss of survival cytokines, or presence of death cytokines), the ratio of pro-apoptotic components (Bax and related molecules) versus anti-apoptotic components (Bcl-2 and related molecules) determines whether the death program will continue to completion. A preponderance of Bax:Bax homodimers promotes continuation of the process while Bax–Bcl-2 heterodimers shuts it down.

PCD = programmed cell death (apoptosis)



■ **FIGURE 2-11** Bcl-2–related proteins and control of apoptosis. Pro-apoptotic (blue ovals) and anti-apoptotic (pink rectangles) Bcl-2–related proteins interact in response to an apoptotic signal. If the pro-apoptotic signals prevail, cytochrome-c (yellow circle) is released from the mitochondria, binds to an adapter protein (Apaf-1), and recruits an initiator caspase (procaspase-9); the resulting caspase-activating assembly, the apoptosome, is associated with the intrinsic pathway of apoptosis.

The cell receives and processes *death signals* from a variety of sources. They converge on this rheostat, which determines whether the cell will activate effector caspases and subsequently whether there will be cleavage of the *death substrates* necessary for apoptosis.[34]

Cells utilize a variety of safeguards to prevent inappropriate apoptosis. They possess a number of proteins that modulate cell death by binding to activated caspases and interfering with their activity, the so-called **i**nhibitors of **a**poptosis **p**roteins (IAPs).[27,28] Some viruses contain viral proteins that perform the same function (e.g., cowpox viral protein CrmA, Adenovirus E1B, Baculovirus p35). These viral proteins block the apoptosis-activating defense of the host cell against viral replication (i.e., block apoptosis).

Apoptosis and the Hematopoietic System

Apoptosis is important in the hematopoietic system (Table 2-6 ★). The default cellular status of hematopoietic precursor cells is *cell death* (Chapter 4). Cytokines and components of the extracellular matrix function to suppress apoptosis, allowing survival of hematopoietic cells when appropriate cytokines are present. Apoptosis plays an essential role in the selection of appropriate recognition repertoires of T and B lymphocytes, eliminating those with nonfunctional or autoreactive antigen receptors (Chapter 8). Apoptosis helps regulate the overall number of mature cells by inducing elimination (cell death) of excess cells when expanded numbers of mature cells are no longer needed (i.e., expanded T- and B-cell clones following elimination of foreign antigen and elimination of neutrophils, eosinophils, and monocytes following an infection/inflammatory response). Apoptosis is the mechanism employed in cytotoxic killing by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Finally, activation of apoptotic caspases is involved in platelet production and release from mature megakaryocytes and in the final stages of erythrocyte maturation (chromatin condensation and organelle removal).[37,38]

Dysregulation of apoptosis also contributes to hematologic disorders. Apoptosis is increased in the myelodysplastic syndromes and tends to be decreased in the acute leukemias, perhaps partly explaining the pancytopenias and leukocytosis, respectively, seen in those disorders (Chapters 25–27).

☑ CHECKPOINT 2-5

What would be the effect on the hematopoietic system homeostasis if the expanded clone of antigen-activated B lymphocytes failed to undergo apoptosis after the antigenic challenge was removed?

★ **TABLE 2-6** The Role of Apoptosis in the Hematopoietic and Lymphoid Systems

I. Default cellular status for hematopoietic stem cells and progenitor cells
Apoptosis regulated by cytokines and extracellular matrix
II. Lymphoid homeostasis
Selection of recognition repertoires of T and B cells
Elimination of autoreactive lymphocytes
Downregulation of immune response following antigen stimulation
Cytotoxic killing by CTL and NK cells
III. Elimination of eosinophils, monocytes, and neutrophils following infection/inflammatory response
IV. Developmental pathways for erythropoiesis and thrombopoiesis

CTL = cytotoxic T lymphocytes; NK = natural killer

ABNORMAL TISSUE HOMEOSTASIS AND CANCER

In recent years, our knowledge of cancer cell biology has exploded. Of significance is the recognition that scattered throughout our own genome are genes that have the potential to cause cancer (**proto-oncogenes**)[39,40] and other genes that have the power to block it (**anti-oncogenes** or **tumor suppressor genes**).[41] As researchers have worked to understand the function of these oncogenes and tumor suppressor genes, they have found that many of them are molecules that regulate normal cell growth and differentiation and/or apoptosis[42–44] (Chapter 23).

Summary

The cell is an intricate, complex structure bound by a membrane. The membrane is a phospholipid bilayer with integral proteins throughout and containing receptors that bind extracellular molecules and transmit messages to the cell's nucleus. Within the cell is the cytoplasm with numerous organelles and the nucleus. The cellular organelles include ribosomes, endoplasmic reticulum, the Golgi apparatus, lysosomes, mitochondria, microfilaments, and microtubules. The nucleus contains the genetic material, DNA, that regulates all cell functions.

The cell cycle is a highly ordered process that results in the accurate duplication and transmission of genetic information from one cell generation to the next. The cell cycle is divided into four stages: M phase (in which cell division or mitosis takes place), S phase (during which DNA synthesis occurs), and two gap phases, G_1 and G_2. G_0 refers to quiescent cells that are temporarily or permanently out of cycle. The normal cell depends on external stimuli (growth factors) to move it out of G_0 and through G_1. The cell cycle is regulated by a series of protein kinases (Cdks) whose activity is controlled by complexing with a regulatory partner (cyclin). Different cyclins with their associated (and activated) Cdks function at specific stages of the cell cycle. Kinase activity is further modulated by both activating and inactivating phosphorylation of kinase subunits and by specific cell-cycle kinase (Cdk) inhibitors. A series of checkpoint controls or surveillance systems functions to ensure the integrity of the process.

Cells utilize the process of programmed cell death, or apoptosis, as well as proliferation to maintain tissue homeostasis. Apoptosis is a unique form of cell death that can be morphologically and biochemically distinguished from necrosis. Apoptosis plays important roles in the development of the organism, in controlling the number of various types of cells, and as a defense mechanism to eliminate unwanted and potentially dangerous cells. Apoptosis is an active process initiated by the cell and results in the orderly dismantling of cellular constituents. Apoptosis is directed by cysteine proteases called *caspases*. Pro-apoptotic and anti-apoptotic proteins (Bcl-2 family members) and specific protein inhibitors (IAPs, or inhibitors of apoptosis) regulate this process. Apoptosis is triggered by loss of survival factors (survival cytokines or extracellular matrix components), presence of death cytokines, or cell-damaging stress.

The various processes that govern tissue homeostasis—proliferation, differentiation, cytokine regulation, and apoptosis—are highly ordered and tightly regulated. When the regulation of these processes malfunctions, the result can be deregulated cell production. Mutations or epigenetic changes that alter the structure or function of the genes that regulate these processes can result in uncontrolled cell growth and malignancy.

Review Questions

Level I

1. Selective cellular permeability and structural stability are provided by: (Objective 1)
   A. membrane lipids
   B. membrane proteins
   C. ribosomes
   D. the nucleus
2. Rough endoplasmic reticulum is important in: (Objective 1)
   A. synthesizing lipid
   B. synthesizing hormones
   C. synthesizing and assembling proteins
   D. phagocytosis
3. The fundamental subunit of chromatin composed of ~180 base pairs of DNA wrapped around a histone protein is called: (Objective 1)
   A. nucleolus
   B. genome
   C. heterochromatin
   D. nucleosome
4. Condensation of chromosomes occurs during which phase of mitosis? (Objective 3)
   A. anaphase
   B. telophase
   C. prophase
   D. metaphase

5. Cells that have exited the cell cycle and entered a nonproliferative phase are said to be in: (Objective 3)

   A. quiescence

   B. interphase

   C. G_1

   D. G_2

6. The regulatory subunit of the active enzyme complex responsible for regulating passage through the various phases of the cell cycle is: (Objective 3)

   A. cyclin

   B. Cdk

   C. Cdk inhibitor

   D. p21

7. The point in the cell cycle at which cell proliferation (cycling) no longer depends on extracellular signals is: (Objective 4)

   A. G_1

   B. R

   C. G_2

   D. M

8. Programmed cell death (cell suicide) is also known as: (Objective 5)

   A. necrosis

   B. senescence

   C. apoptosis

   D. terminal differentiation

9. All of the following are considered initiators of apoptosis *except*: (Objective 6)

   A. estrogens

   B. death cytokines

   C. loss of matrix attachment

   D. cell-damaging stress

10. Which of the following events in hematopoiesis is regulated by apoptosis? (Objective 7)

    A. removal of excess neutrophils following cessation of bacterial challenge

    B. removal of excess B lymphocytes following immune stimulation

    C. removal of excess platelets following hemostatic challenge

    D. A and B

Level II

1. UTRs are regions of mRNA that: (Objective 1)

   A. represent variations of the genetic sequence of a gene in different individuals

   B. represent the regions of the gene that are transcribed

   C. contain the splice sites for mRNA processing

   D. influence the stability of mRNA and translation of protein

2. The main function of the ubiquitin-proteosome system is to: (Objective 2)

   A. assist in the three-dimensional folding of polypeptides into their tertiary structure

   B. degrade unwanted or damaged polypeptides

   C. facilitate transfer of polypeptides from the endoplasmic reticulum to the Golgi

   D. direct post-translational modifications of proteins

3. The kinase complex responsible for passage through and exit from mitosis is composed of: (Objective 3)

   A. cyclin A/Cdk2

   B. cyclin D/Cdk4

   C. cyclin B/Cdk1

   D. cyclin E/Cdk2

4. CAK, the kinase activity responsible for the activating phosphorylations of Cdks, consists of: (Objective 4)

   A. cyclin A/Cdk1

   B. cyclin H/Cdk7

   C. cyclin F/Cdk6

   D. cyclin C/Cdk2

5. Overexpression of the p21 protein would have what effect on the cell cycle of proliferating cells? (Objective 4)

   A. decrease cell-cycle progression

   B. increase cell-cycle progression

   C. trigger apoptosis

   D. none

6. The protein responsible for binding the transcription factors E2F and blocking cell-cycle progression beyond the restriction point (R) is: (Objective 6)

   A. p53

   B. p15

   C. p21

   D. Rb

7. Apoptotic cell death is characterized by all of the following *except*: (Objective 14)

   A. triggering an inflammatory response

   B. condensation of the nucleus

   C. cleavage of chromatin into discrete fragments (multiples of 185 base pairs)

   D. condensation of the cytoplasm and cell shrinkage

8. The components of apoptosis directly responsible for dismantling the cell during the programmed cell death process are: (Objective 8)

   A. Bcl-2 family members

   B. IAPs

   C. initiator caspases

   D. effector caspases

9. A predominance of Bax-Bax homodimers has what effect on apoptosis? (Objective 11)

   A. inhibits initiator caspases

   B. promotes activation of effector caspases

   C. activates death receptors on the cell surface

   D. neutralizes IAPs

10. Which of the following are associated with gene silencing? (Objective 13)

    A. DNA (CpG) methylation and histone acetylation

    B. DNA (CpG) methylation and histone deacetylation

    C. unmethylated CpG and histone acetylation

    D. unmethylated CpG and histone deacetylation

Companion Resources

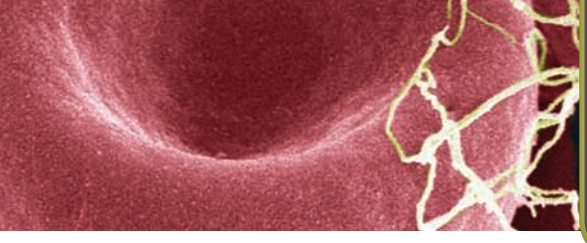
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Singer SJ, Nicholson GL. The fluid mosaic model of the structure of cell membranes. *Science*. 1972;175:720–31.
2. Wagner AJ, Berliner N, Benz EJ. Anatomy and physiology of the gene. In: Hoffman R, Benz EJ, Silberstein LE et al., eds. *Hematology: Basic Principles and Procedures*, 6th ed. New York: Churchill Livingstone; 2013:3–15.
3. Papayannopoulou T, Migliaccio AR. Biology of erythropoiesis, erythroid differentiation, and maturation. In: Hoffman R, Benz EJ, Silberstein LE et al., eds. *Hematology: Basic Principles and Procedures*, 6th ed. New York: Churchill Livingstone; 2013:258–79.
4. Kaufman RJ, Popolo L. Protein synthesis, processing, and trafficking. In: Hoffman R, Benz EJ, Silberstein LE et al., eds. *Hematology: Basic Principles and Procedures*, 6th ed. New York: Churchill Livingstone; 2013:35–47.
5. Lecker SH, Saric T, Goldberg AL. Protein degradation in cells. In: Hoffman R, Benz EJ, Shattil SJ et al., eds *Hematology: Basic Principles and Procedures*, 5th ed. New York: Churchill Livingstone; 2009:34-41.
6. Pardee AB. G_1 events and regulation of cell proliferation. *Science*. 1989;246:603–8.
7. Nasmyth K. Viewpoint. Putting the cell cycle in order. *Science*. 1996;274:1643–45.
8. Sherr CJ. G_1 phase progression: Cycling on cue. *Cell*. 1994;79:551–55.
9. King RW, Deshaies RJ, Peters JM, Kirschner MW. How proteolysis drives the cell cycle. *Science*. 1996;274:1652–59.
10. Morgan DO. Principles of Cdk regulation. *Nature*. 1995;374:131–34.
11. Sherr CJ, Roberts JM. Inhibitors of mammalian G-1 cyclin dependent kinases. *Genes Develop*. 1995;9:1149–63.
12. Murray AW. Creative blocks: Cell cycle checkpoints and feedback controls. *Nature*. 1992;359:599–604.
13. Gorbsky GJ. Cell cycle checkpoints: Arresting progress in mitosis. *BioEssays*. 1997;19:193–97.
14. Nurse P. Checkpoint pathways come of age. *Cell*. 1997;91:865–67.
15. Lee WM, Dang CV. Control of cell division. In: Hoffman R, Benz EJ, Silberstein LE et al., eds. *Hematology: Basic Principles and Procedures*. 6th ed. New York: Churchill Livingstone; 2013:147–57.
16. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995;81:323–30.
17. Herwig S, Strauss M. The retinoblastoma protein: a master regulator of cell cycle, differentiation, and apoptosis. *Eur J Biochem*. 1997;246:581–601.
18. Sidransky D, Hollstein M. Clinical implications of the p53 gene. *Ann Rev Med*. 1996;47:285–301.
19. Jorde LB. Genomics and Epigenetics. In: Prchal JT, Kaushansky K, Lichtman MA, Kipps TJ, Seligsohn U, eds. *Williams Hematology*. 8th ed. New York: McGraw-Hill; 2010. http://www.accessmedicine.com/content.aspx?aID=6127928. Accessed October 11, 2012.
20. Ordway JM, Curran T. Methylation matters: modeling a manageable genome. *Cell Growth Diff*. 2002;13:149–162.
21. Grunstein M. Histone acetylation in chromatin structure and transcription. *Nature*. 1997;389:349–52.
22. Burger M. Hyperacetylated chromatin domains: lessons from heterochromatin. *J Biol Chem*. 2005;280:21689–92.
23. Kerr JFR, Harmon BV. Definition and incidence of apoptosis: an historical perspective. In: *Apoptosis: The Molecular Basis of Cell Death*. New York: Cold Spring Harbor Laboratory Press; 1991:5–25.
24. Steller H. Mechanisms and genes of cellular suicide. *Science*. 1995;267:1445–49.
25. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science*. 1995;267:1456–62.
26. Hetts SW. To die or not to die: an overview of apoptosis and its role in disease. *JAMA*. 1998;279:300–7.
27. Thornberry NA, Lazebik Y. Caspases: enemies within. *Science*. 1998;281:1312–16.

28. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Ann Rev Biochem*. 1999;68:383–424.
29. Salvesen GS, Dixit VM. Caspase activation: the induced proximity model. *Proc Natl Acad Sci USA*. 1999;96:10:964–67.
30. Nagata S, Golstein P. The Fas death factor. *Science*. 1995;267:1449–56.
31. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science*. 1998;281:1305–8.
32. Danial NN, Hockenbery DM. Cell death. In: Hoffman R, Benz EJ, Silberstein LE et al., eds. *Hematology: Basic Principles and Procedures*. 6th ed. New York: Churchill Livingstone; 2013:158–69.
33. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science*. 1998;281:1322–26.
34. Chao DT, Korsmeyer SJ. Bcl-2 family: regulators of cell death. *Ann Rev Immunol*. 1998;16:395–419.
35. Susin SA, Zamzami N, Kroemer G. Mitochondria as regulators of apoptosis: Doubt no more. *Biochem Biophys Acta*. 1998;1366:151–65.
36. Green DR, Reed JC. Mitochondria and apoptosis. *Science*. 1998;281:1309–12.
37. Kaluzhny Y, Ravid K. Role of apoptotic processes in platelet biogenesis. *Acta Haematol*. 2004;111:67–77.
38. Kolbus A, Pilat S, Huszk A. et al. Raf-1 antagonizes erythroid differentiation by restraining caspase activation, *J Exp Med*. 2002;196: 1347–53.
39. Prochownik E. Protooncogenes and cell differentiation. *Trans Med Rev*. 1989;3:24–38.
40. Studzinski GP. Oncogenes, growth, and the cell cycle: an overview. *Cell Tissue Kinet*. 1989;22:405–24.
41. Carbone DP, Minna JD. Antioncogenes and human cancer. *Ann Rev Med*. 1993;44:451–64.
42. Marx J. How cells cycle toward cancer. *Science*. 1994;263:319–21.
43. Marx J. Learning how to suppress cancer. *Science*. 1993;261:1385–87.
44. Rinkenberger JL, Korsmeyer SJ. Errors of homeostasis and deregulated apoptosis. *Curr Opin Genet Dev*. 1997;7:589–96.



3 Structure and Function of Hematopoietic Organs

ANNETTE J. SCHLUETER, MD, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Identify the sites of hematopoiesis during embryonic and fetal development, childhood, and adulthood.
2. Identify organ/tissue sites in which each hematopoietic cell type differentiates.
3. Explain the difference between primary and secondary lymphoid tissue.
4. Describe the function of bone marrow, spleen, lymph nodes, and thymus.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Associate physical findings (hypersplenism, lymphadenopathy) with the presence of hematologic disease.
2. Assess the pathophysiologic changes that lead to bone marrow hyperplasia or extramedullary hematopoiesis.
3. Identify sites of extramedullary hematopoiesis.
4. Create a sketch of the structure of bone marrow, spleen, lymph nodes, and thymus that shows the location of hematopoietic cells.
5. Differentiate between primitive and definitive erythropoiesis.

Key Terms

Adipocyte
Blood island
Culling
Endosteum
Erythroblastic island
Extramedullary hematopoiesis
Germinal center
Hematopoietic stem cell (HSC)
Hyperplasia
Lymphoid follicle
Medullary hematopoiesis
Mesenchymal stem cell (MSC)
Osteoblast
Osteoclast
Pitting
Reticular cell
Stroma
Trabecula

Chapter Outline

Background Basics

The information in this chapter builds on the concepts learned in the first chapter. A basic anatomy and physiology course could also be helpful. To maximize your learning experience, you should review these concepts before starting this unit of study:

LEVEL I AND LEVEL II

- List the types of blood cells and give their basic functions
- Define *hemoglobin* and explain its function
- Locate the tables in the book that give the reference intervals for blood cells and hemoglobin

CASE STUDY

We will refer to this case throughout this chapter.

Francine, a 10-year-old female, was brought to her pediatrician for complaints of lethargy and leg pain. Physical examination revealed splenomegaly and lymphadenopathy. A complete blood count was ordered with the following results: Hb 8 g/dL; WBC 6.5 $\times$ 10^9/L; platelets 21 $\times$ 10^9/L.

Refer to the tables on the front inside cover of the book and determine which blood cell parameters, if any, are abnormal.

OVERVIEW

This chapter includes a description of the tissues involved in the production and maturation of blood cells. It begins with a look at blood cell production sequentially from the embryo to the adult. Each tissue's histologic structure and its function in hematopoiesis are discussed. Abnormalities in hematopoiesis that are associated with histologic and functional changes in these tissues are briefly described.

INTRODUCTION

Cellular proliferation, differentiation, and maturation of blood cells take place in the hematopoietic tissue, which in the adult consists primarily of bone marrow, although some lymphocyte development also takes place in the spleen and thymus. Mature cells are released to the peripheral blood and can live out their lifespan in the blood or take up residence in the spleen, lymph nodes, or other tissues. The link between the bone marrow and blood cell production was not established until it was recognized that blood formation was a continuous process. Before 1850, it was believed that blood cells formed in the fetus were viable until the host's death and that there was no need for a continuous source of new elements.

DEVELOPMENT OF HEMATOPOIESIS

Hematopoiesis begins as early as the eighteenth day after fertilization in an extraembryonic location, the yolk sac of the human embryo.[1] The cells made in the yolk sac include erythrocytes and a few macrophages.[2] The ability to make erythrocytes is important because the embryo must be able to transport oxygen to developing tissue early in gestation. Shortly thereafter, at about 4 weeks of gestation, intraembryonic hematopoiesis begins in the aorta-gonad-mesonephros (AGM) region located in the ventral lumen of the developing aorta. This region has the ability to make a wider range of hematopoietic cells, including lymphocytes, than those made in the yolk sac.[3] Erythrocyte production from the yolk sac is called *primitive erythropoiesis*. Hemoglobin in these cells is not typical of that seen in later developing erythroblasts, they do not differentiate from self-renewing **hematopoietic stem cells (HSCs)**, and they complete their maturation in the circulation rather than in an organ.[4] The yolk sac erythroblasts arise from clusters of cells called **blood islands** and are closely related to development of endothelium, the cells lining blood vessels.[5] *Definitive erythropoiesis* begins with the formation of self-renewing HSC in the AGM. The HSC is the common precursor cell for all developing hematopoietic cells and is characterized by its ability to proliferate without differentiation ("self-renewal"). Primitive erythroblasts have a megaloblastic appearance (large cells with coarse clumped chromatin; Chapter 15). The hemoglobin in these cells consists of the embryonic varieties, Gower 1, Gower 2, and Portland[6] (Chapter 6).

At about the third month of fetal life, the liver becomes the chief site of blood cell production by which time the yolk sac and AGM have discontinued their role in hematopoiesis. The liver continues to produce a high proportion of erythroid cells, but myeloid and lymphoid cells begin to appear in greater numbers,[7] indicating the beginning of a transition to adult patterns of hematopoiesis in which myeloid differentiation predominates over erythroid differentiation. Hemoglobin F production replaces the embryonic hemoglobins during this period.

As fetal development progresses, hematopoiesis also begins to a lesser degree in the spleen, kidney, thymus, and lymph nodes. Erythroid and myeloid cell production as well as early B lymphocyte (a subclass of lymphocytes) development gradually shifts from these sites to bone marrow during fetal and neonatal life as the hollow cavities within the bones are formed. The bone marrow becomes the primary site of hematopoiesis at about the sixth month of gestation and

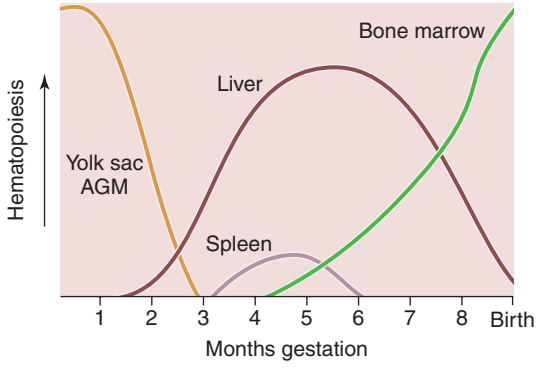


FIGURE 3-1 Location of hematopoiesis during fetal development. At birth, most blood cell production is limited to the marrow.

continues as the primary source of blood production after birth and throughout adult life (Figure 3-1 ■). Granulocyte and megakaryocyte (precursor of platelets) production shifts to the bone marrow before erythropoiesis, which does not transition until the end of gestation. The thymus becomes the major site of T lymphocyte (a subclass of lymphocytes) production during fetal development and continues to be active throughout the neonatal period and childhood. As is true for erythrocytes in the yolk sac, the first T cells to develop differ from their adult counterparts. They use a different set of genes to make the T cell receptor, which the T cell uses to recognize and react to foreign substances[8] (Chapter 8). Lymph nodes and the spleen continue as an important site of late B-cell differentiation throughout life.

HEMATOPOIETIC TISSUE

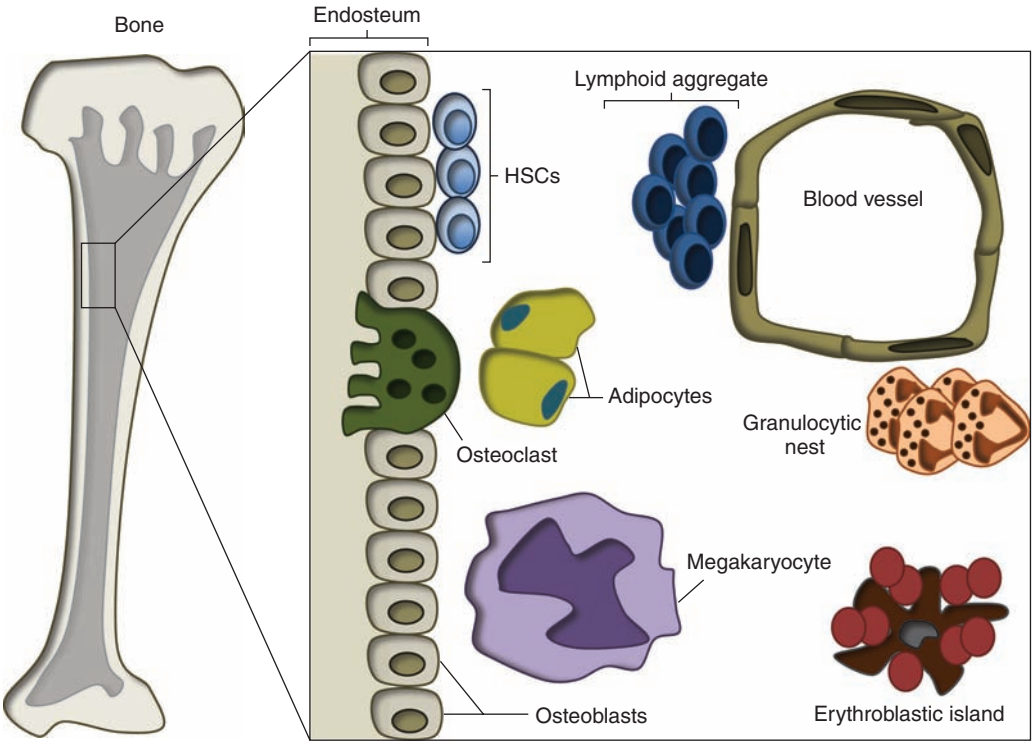
The adult hematopoietic system includes tissues and organs involved in the proliferation, maturation, and destruction of blood cells. These organs and tissues include the bone marrow, thymus, spleen, and lymph nodes. Bone marrow is the site of myeloid, erythroid, and megakaryocyte as well as early stages of lymphoid cell development. Thymus, spleen, and lymph nodes are primarily sites of later lymphoid cell development. Tissues in which lymphoid cell development occurs are divided into primary and secondary lymphoid tissue. *Primary lymphoid tissues* (bone marrow and thymus) are those in which T and B cells develop from nonfunctional precursors into cells capable of responding to foreign antigens (immunocompetent cells). *Secondary lymphoid tissues* (spleen and lymph nodes) are those in which immunocompetent T and B cells further divide and differentiate into effector cells and memory cells in response to antigens (Chapter 8).

Bone Marrow

Blood-forming tissue located between the **trabeculae** of spongy bone is known as *bone marrow*. (*Trabecula* refers to a projection of bone extending from cortical bone into the marrow space; it provides support for marrow cells.) This major hematopoietic organ is a cellular, highly vascularized, loose connective tissue. It is composed of two major compartments: the vascular and the endosteal. The vascular compartment is composed of the bone marrow arteries and veins, stromal cells, and hematopoietic cells (Figure 3-2 ■). The endosteal compartment is primarily the site of bone remodeling but also contains HSC.

Vasculature

The vascular supply of bone marrow is served by two arterial sources, a nutrient artery and a periosteal artery, that enter the bone through small holes, the bone foramina. Blood is drained from the marrow via the central vein (Figure 3-3 ■). The nutrient artery branches around the central sinus that spans the marrow cavity. Arterioles radiate outward from the nutrient artery to the **endosteum** (the inner lining of the cortical bone), giving rise to capillaries that merge with capillaries from periosteal arteries to form sinuses within the marrow. The sinuses, lined by single endothelial cells and supported on the abluminal side (away from the luminal surface) by adventitial **reticular cells**, ultimately gather into wider collecting sinuses, which open into the central longitudinal vein.[9] The central longitudinal vein continues through the length of the marrow and exits through the foramen where the nutrient artery entered. Nerve fibers surrounding marrow arteries regulate blood flow into



■ **FIGURE 3-2** Schematic drawing of a section of bone marrow.
Figure courtesy of Dr. Corey Parlet.

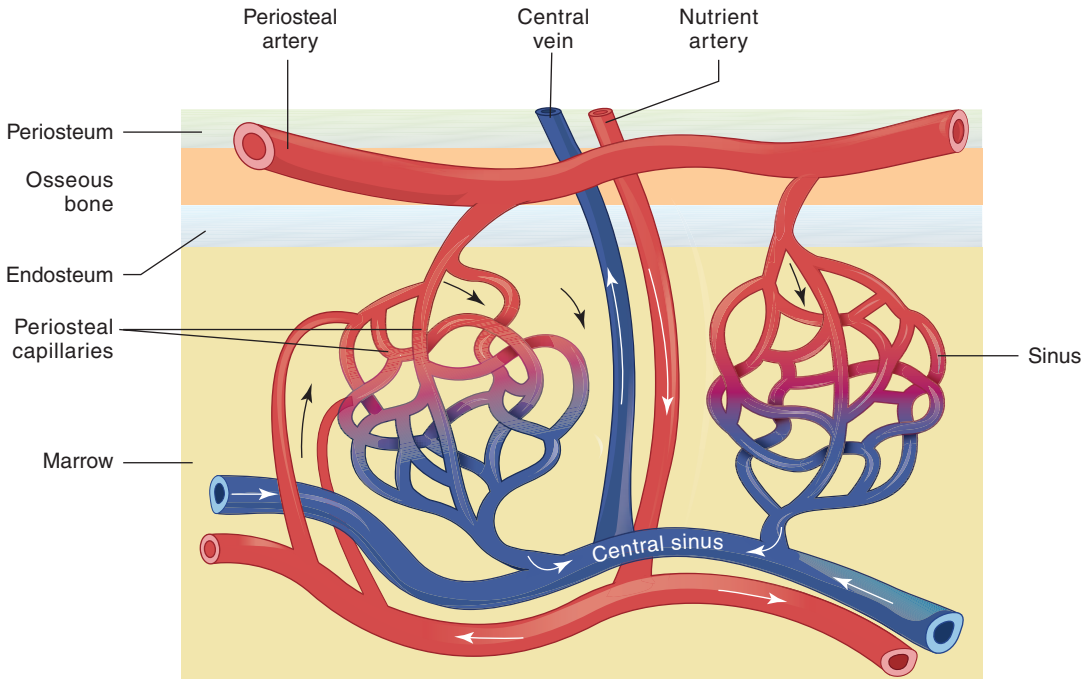


FIGURE 3-3 Diagram of the microcirculation of bone marrow. The major arterial supply to the marrow is from periosteal capillaries and capillary branches of the nutrient artery that have traversed the bony enclosure of the marrow through the bone foramina. The capillaries join with the venous sinuses as they re-enter the marrow. The sinuses gather into wider collecting sinuses that then open into the central longitudinal vein (central sinus).

the bone marrow, which in turn controls hematopoietic progenitor release into the circulation.[10]

Stroma

The bone marrow **stroma** (supporting tissue in the vascular compartment) provides a favorable microenvironment for sustained proliferation of hematopoietic cells, forming a meshwork that creates a three-dimensional scaffolding for them.[11] Stromal cellular components also provide cytokines that regulate hematopoiesis (Chapter 4). The stroma is composed of three major cell types: macrophages, reticular cells (fibroblasts), and **adipocytes** (fat cells).

Macrophages serve two major functions in the bone marrow: phagocytosis and secretion of hematopoietic cytokines (proteins secreted by a cell; these proteins modulate the function of another cell). Macrophages phagocytose the extruded nuclei of maturing erythrocytes, B cells that have not differentiated properly, and differentiating cells that die during development. Some macrophages serve as the center of the erythroblastic islands as discussed in the section "Hematopoietic Cells." Macrophages also provide many colony-stimulating factors (cytokines that stimulate the growth and development of immature hematopoietic cells) needed for the development of myeloid lineage cells. Macrophages stain acid phosphatase positive.

Reticular cells are located on the abluminal surface of the vascular sinuses and send long cytoplasmic processes into the stroma. They are an abundant source of CXCL12 (SDF-1), which is critical for maintaining an HSC pool in the marrow.[12] These cells also produce reticular fibers, which contribute to the three-dimensional supporting network that holds the vascular sinuses and hematopoietic elements. The fibers can be visualized with light microscopy and after silver staining. Reticular cells are alkaline phosphatase positive.

Adipocytes are cells whose cytoplasm is largely replaced with a single fat vacuole. They differentiate from **mesenchymal stem cells (MSCs)**, and their production is inversely proportional to osteoblast formation.[13] MSCs are multipotent stromal cells that can differentiate into bone, cartilage, and fat cells. Adipocytes mechanically control the volume of bone marrow in which active hematopoiesis takes place. They also provide steroids and other cytokines that influence hematopoiesis and maintain osseous bone integrity.[14,15]

The proportion of bone marrow composed of adipocytes changes with age. For the first 4 years of life, nearly all marrow cavities are composed of hematopoietic cells, or red marrow. After 4 years of age, adipocytes or yellow marrow gradually replaces the red marrow in the shafts of long bones. By the age of 25 years, hematopoiesis is limited to the marrow of the skull, ribs, sternum, scapulae, clavicles, vertebrae, pelvis, upper half of the sacrum, and proximal ends of the long bones. The distribution of red:yellow marrow in these bones is about 1:1. The fraction of red marrow in these areas continues to decrease with aging.

Osteoblasts and osteoclasts are found in the endosteum (internal surface of calcified bone). These cells can be dislodged during bone marrow biopsy and can be found in the specimen with hematopoietic cells. Osteoblasts differentiate from MSCs; osteoclasts differentiate from HSCs.[16]

Osteoblasts are involved in the formation of calcified bone and produce cytokines that can positively or negatively regulate HSC

activity.[17] They are large cells (up to 30 mcM (μm) in diameter) that resemble plasma cells except that the perinuclear halo (Golgi apparatus) is detached from the nuclear membrane and, in Wright-stained specimens, appears as a light area away from the nucleus (Figure 3-4a ■). In addition, the cytoplasm can be less basophilic, and the nucleus has a finer chromatin pattern than plasma cells. Osteoblasts are normally found in groups and are more commonly seen in children and in metabolic bone diseases. The cells are alkaline phosphatase positive.

Osteoclasts are cells related to macrophages that are involved in resorption and remodeling of calcified bone. Up to 100 mcM in diameter, they are even larger than osteoblasts. The cells are multinucleated, form from fusion of activated monocytes, and have granular cytoplasm that can be either acidophilic or basophilic. They resemble megakaryocytes except that the nuclei are usually discrete (whereas the megakaryocyte has a single, large multilobulated nucleus) and often contain nucleoli (Figure 3-4b ■).

Hematopoietic Cells

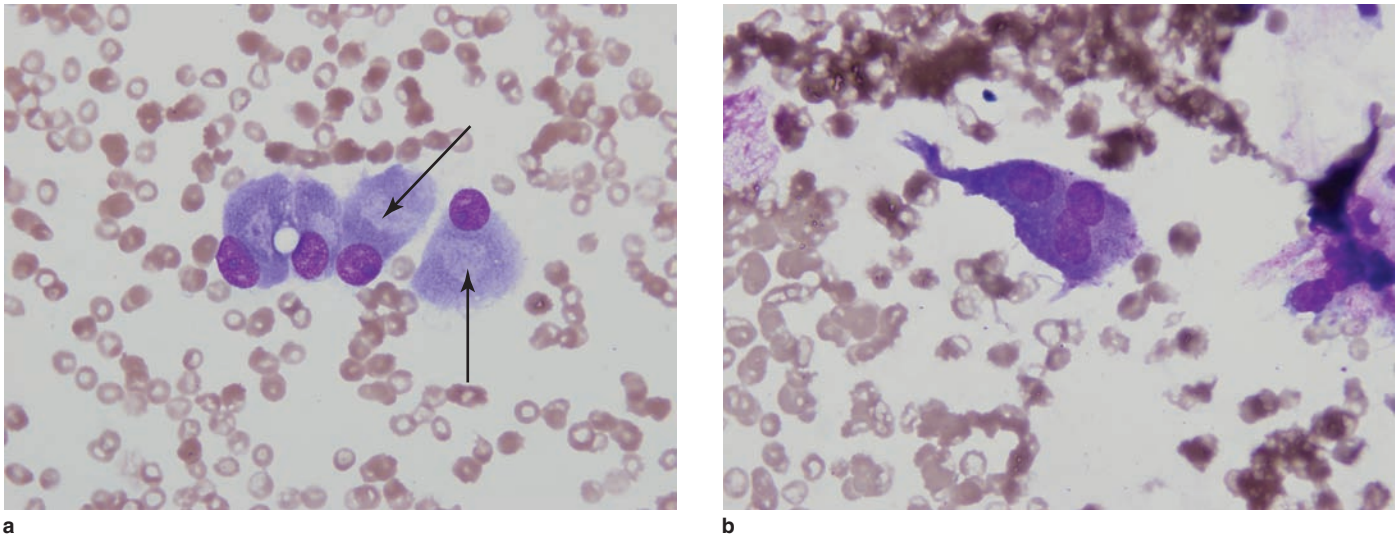
These cells are arranged in distinct niches within the vascular compartment of the marrow cavity. Erythroblasts constitute 25–30% of the marrow cells and are produced near the venous sinuses. They develop in **erythroblastic islands** composed of a single macrophage surrounded by erythroblasts in varying states of maturation. The macrophage cytoplasm extends out to surround the erythroblasts. During this close association, the macrophages regulate erythropoiesis by secreting various cytokines.[18] The least mature cells are closest to the center of the island, and the more mature cells are at the periphery.

The location of leukocyte development differs depending on the cell type. Granulocytes are produced in nests close to the trabeculae and arterioles and are relatively distant from the venous sinuses. These nests are not quite as apparent morphologically as are erythroblastic islands. Megakaryocytes are very large, polyploid cells (DNA content more than 2N) that produce platelets from their cytoplasm. They are located adjacent to the vascular sinus.[19] Cytoplasmic processes of the megakaryocyte form long proplatelet processes that pinch off to form platelets. Lymphocytes are normally produced in lymphoid aggregates located near arterioles. Lymphoid progenitor cells can leave the bone marrow and travel to the thymus where they mature into T lymphocytes. Some remain in the bone marrow where they mature into B lymphocytes. Some B cells return to the bone marrow after being activated in the spleen or lymph node. Activated B cells transform into plasma cells, which can reside in the bone marrow and produce antibody.

☑ CHECKPOINT 3-1

Describe the bone marrow stromal location of erythrocyte, granulocyte, platelet, and lymphocyte differentiation.

Bone forms a rigid compartment for the marrow. Thus, any change in volume of the hematopoietic tissue, as occurs in many anemias and leukemias, must be compensated for by a change in the space-occupying adipocytes. Normal red marrow can respond to stimuli and increase its activity to several times the normal rate. As a result, the red marrow becomes hyperplastic and replaces portions of the fatty marrow. Bone marrow **hyperplasia** (an excessive proliferation of normal cells) accompanies all conditions with increased or ineffective hematopoiesis. The degree of hyperplasia is related to the severity and duration of the pathologic state. Acute blood loss can cause erythropoietic tissue to temporarily replace fatty tissue; severe chronic anemia can cause erythropoiesis to be so intense that it not only replaces fatty marrow but also erodes the bone's internal surface. In malignant diseases that invade or originate in the bone marrow such as leukemia, proliferating abnormal cells can replace both normal hematopoietic tissue and fat.



a b

■ **FIGURE 3-4** (a) Osteoblast; arrows point to the Golgi apparatus (perinuclear halo). (b) Osteoclast in bone marrow aspirate. (Wright-Giemsa stain; 1000× magnification.)

In contrast, the hematopoietic tissue can become inactive or hypoplastic (a condition in which the hematopoietic cells in bone marrow decrease). Fat cells then increase, providing a cushion for the marrow. Environmental factors such as chemicals and toxins can suppress hematopoiesis whereas other types of hypoplasia can be genetically determined (Chapter 16). Myeloproliferative disease, which begins as a hypercellular disease, frequently terminates in a state of aplasia (absence of hematopoietic tissue in bone marrow) in which fibrous tissue replaces hematopoietic tissue (Chapter 25).

CASE STUDY (continued from page 26)

Microscopic examination of a stained blood smear from Francine revealed a predominance of very young blood cells (blasts) in the peripheral blood. These cells are normally found only in the bone marrow. Subsequently, she had bone marrow aspirated for examination. This revealed 100% cellularity (red marrow) with a predominance of the same type of blasts as those found in the peripheral blood.

1. Describe Francine's bone marrow as normal, hyperplastic, or hypoplastic.
2. What conditions can cause this bone marrow finding?

Blood Cell Egress

Special properties of the maturing hematopoietic cell and of the venous sinus wall are important in migration of blood cells from the bone marrow to the circulation.[20] These cells must migrate *between* reticular cells but *through* endothelial cells to reach the circulation. As cell traffic across the sinus increases, the reticular cells contract, creating a less continuous layer over the abluminal sinus wall. When the reticular cell layer contracts, it creates compartments between the reticular cell layer and the endothelial layer where mature cells accumulate and can interact with sites on the sinus endothelial surface.

The new blood cell interacts with the abluminal endothelial membrane by a receptor-mediated process, forcing the abluminal membrane into contact with the luminal endothelial membrane. The two membranes fuse, and under pressure from the passing cell, they separate, creating a pore through which the hematopoietic cell enters the lumen of the sinus. These pores are only 2–3 mcM in diameter; thus, blood cells must have the ability to deform so that they can pass through the sinusoidal lining. Progressive increases in deformability and motility have been noted as granulocytes mature from the myeloblast to the segmented granulocyte stage, facilitating the movement of cells into the sinus lumen.

Many soluble factors are important in regulating the release of blood cells from bone marrow, including granulocyte-colony stimulating factor (G-CSF), granulocyte monocyte-colony stimulating factor (GM-CSF), and a large number of chemokines[21,22] (Chapter 4). Some of these molecules are used clinically to increase circulating granulocytes or release HSCs into the circulation to obtain granulocytes for transfusion or stem cells for transplantation.

☑ CHECKPOINT 3-2

Describe the process by which a blood cell moves from the marrow to the vascular space.

Extramedullary Hematopoiesis

Hematopoiesis in the bone marrow is called **medullary hematopoiesis**. **Extramedullary hematopoiesis** denotes blood cell production in hematopoietic tissue other than bone marrow. In certain hematologic disorders, when hyperplasia of the marrow cannot meet the physiologic blood needs of the tissues, extramedullary hematopoiesis can occur in the hematopoietic organs that were active in the fetus, principally the liver and spleen. Organomegaly frequently accompanies significant hematopoietic activity at these sites.

Thymus

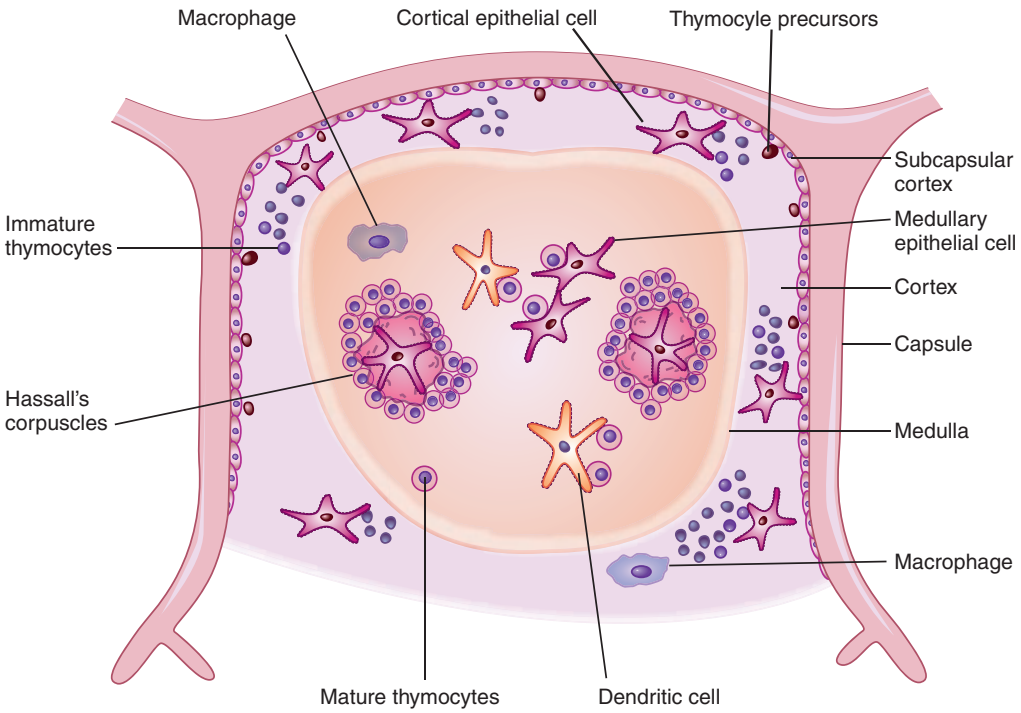
The thymus is a lymphopoietic organ located in the upper part of the anterior mediastinum. It is a bilobular organ demarcated into an outer cortex and central medulla. The cortex is densely packed with small lymphocytes (thymocytes), cortical epithelial cells, and a few macrophages. The medulla is less cellular and contains more mature thymocytes mixed with medullary epithelial cells, dendritic cells, and macrophages (Figure 3-5 ■).

The primary purpose of the thymus is to serve as a compartment in which T lymphocytes mature.[23] Precursor T cells leave the bone marrow and enter the thymus through arterioles in the cortex. As they travel through the cortex and the medulla, they interact with epithelial cells and dendritic cells, which provide signals to ensure that T cells can recognize foreign antigen but not self-antigen. They also undergo rapid proliferation. Only about 3% of the cells generated in the thymus successfully exit the medulla as mature T cells; the rest die by apoptosis and are removed by thymic macrophages. The thymus is responsible for supplying the T-dependent areas of lymph nodes, spleen, and other peripheral lymphoid tissue with immunocompetent T lymphocytes.

The thymus is a well-developed organ at birth and continues to increase in size until puberty. After puberty, however, it begins to atrophy until old age when it becomes barely recognizable. This atrophy may be driven by increased steroid levels beginning in puberty and decreased growth factor levels in adults.[24,25] The atrophy is characterized by reduced expression of a transcription factor (FOXN1) required for thymic epithelial cell differentiation.[26] The atrophied thymus is still capable of producing some new T cells if the peripheral pool becomes depleted as occurs after the lymphoid irradiation that accompanies bone marrow transplantation.[27]

Spleen

The spleen is located in the upper-left quadrant of the abdomen beneath the diaphragm and to the left of the stomach. After several emergency splenectomies were performed without causing permanent harm to the patients, it was recognized that the spleen was not essential to life. However, it does play a role in filtering foreign substances and old erythrocytes from the circulation, storage of platelets, and immune defense.



■ **FIGURE 3-5** A schematic drawing of the thymus. See text for the role of the various cell types. Hassall's corpuscles are collections of epithelial cells that may be involved in the development of certain T-cell subsets (regulatory T cells) in the thymus.

Architecture

Enclosed by a capsule of connective tissue, the spleen contains the largest collection of lymphocytes and macrophages in the body (Figure 3-6 ■). These cells, together with a reticular meshwork, are organized into three zones: white pulp, red pulp, and the marginal zone.

The white pulp, a visible grayish-white zone, is composed of lymphocytes and is located around a central artery. The area closest to the artery, which contains many T cells as well as macrophages and dendritic cells, is termed the *periarteriolar lymphatic sheath (PALS)*. Peripheral to this area are B cells arranged into follicles (a sphere of B cells within lymphatic tissue). Activated B cells are found in specialized follicular areas called **germinal centers**, which appear as lightly stained areas in the center of a **lymphoid follicle**. The germinal centers consist of a mixture of B lymphocytes, follicular dendritic cells, and phagocytic macrophages. The immune response is initiated in the white pulp. In some cases of heightened immunologic activity, the white pulp can increase to occupy half the volume of the spleen (it is normally ≤20%).

White pulp is surrounded by the marginal zone, a reticular meshwork containing blood vessels, macrophages, and specialized B cells. This zone lies at the junction of the white pulp and red pulp and is important in initiating rapid immune responses to blood-borne pathogens and performing functions similar to that of the red pulp.

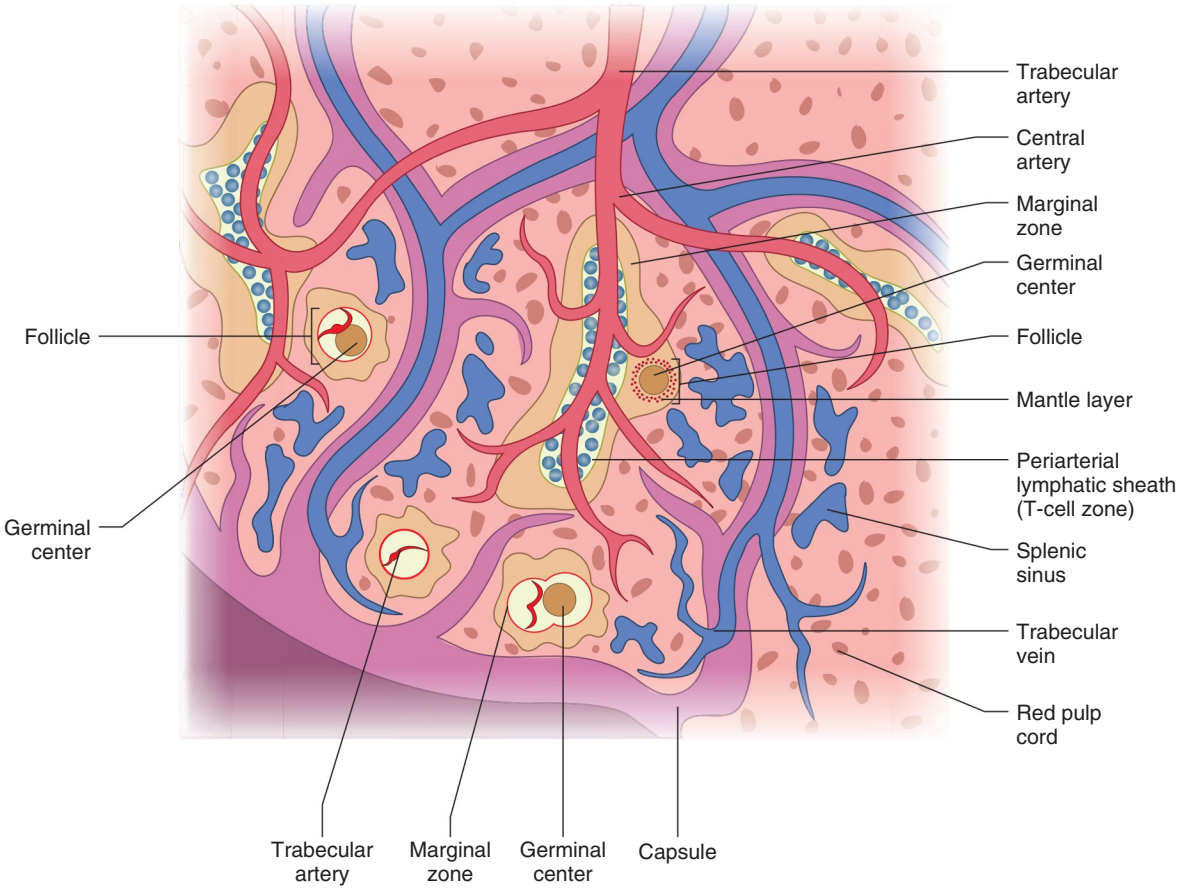
The red pulp contains sinuses and cords. The sinuses are dilated vascular spaces for venous blood. The pulp's red color is caused by the presence of large numbers of erythrocytes in the sinuses. The cords are composed of masses of reticular tissue and macrophages that lie between the sinuses. The cords of the red pulp provide zones for platelet storage and destruction of damaged blood cells.

Blood Flow

The spleen is richly supplied with blood. It receives 5% of the total cardiac output, a blood volume of 300 mL/minute. Blood enters the spleen through the splenic artery, which branches into many central arteries. Vessel branches can terminate in the white pulp, red pulp, or marginal zone. Blood entering the spleen can follow either the rapid transit pathway (closed circulation) or the slow transit pathway (open circulation). The rapid transit pathway is a relatively unobstructed route by which blood enters the sinuses in red pulp from the arteries and passes directly to the venous collecting system. In contrast, blood entering the slow transit pathway moves sluggishly through a circuitous route of macrophage-lined cords before it gains access to the venous sinuses. Plasma in the cords freely enters the sinuses, but erythrocytes meet resistance at the sinus wall where they must squeeze through the tiny openings. This skimming of the plasma from blood cells sharply increases the hematocrit in the cords. Sluggish blood flow and continued erythrocyte metabolic activity in the cords result in a splenic environment that is hypoxic, acidic, and hypoglycemic. Hypoxia and hypoglycemia occur as erythrocytes utilize available oxygen and glucose, and metabolic byproducts create the acidic environment.

Function

Blood that empties into the cords of the red pulp or the marginal zone takes the slow transit pathway, which is very important to splenic function including culling, pitting, and storing blood cells. The discriminatory filtering and destruction of senescent (aged) or damaged red cells by the spleen is termed **culling**. Cells entering the spleen through the slow transit pathway become concentrated in the hypoglycemic, hypoxic cords of the red pulp—a hazardous environment for aged



■ **FIGURE 3-6** A schematic drawing of splenic tissue. See text for an explanation of the splenic tissue architecture. The periarterial lymphatic sheath contains many T cells, macrophages, and dendritic cells. B cells are arranged into follicles. Activated B cells are in the germinal centers.

or damaged erythrocytes. Slow passage through a macrophage-rich route allows the phagocytic cells to remove these old or damaged, less deformable erythrocytes before or during their squeeze through the 3 mcM pores to the venous sinuses. Normal erythrocytes withstand this adverse environment and eventually re-enter the circulation.

Pitting refers to the spleen's ability to "pluck out" particles from intact erythrocytes without destroying them. Blood cells coated with antibody are susceptible to pitting by macrophages. The macrophage removes the antigen–antibody complex and the attached membrane. The pinched-off cell membrane can reseal itself, but the cell cannot synthesize lipids and proteins for new membrane due to its lack of cellular organelles. Therefore, extensive pitting causes a reduced surface-area-to-volume ratio, resulting in the formation of spherocytes (erythrocytes that have no area of central pallor on stained blood smears). The presence of spherocytes on a blood film is evidence that the erythrocyte has undergone membrane assault in the spleen.

☑ CHECKPOINT 3-3

Describe how the spleen removes old or damaged erythrocytes from the circulation.

The white pulp and marginal zones of the spleen are important lines of defense in blood-borne infections because of their rich supply of lymphocytes and phagocytic cells (macrophages) and the slow transit circulation through these areas. Blood-borne antigens are forced into close contact with macrophages (functioning as antigen-presenting cells) and lymphocytes allowing for recognition of the antigen as foreign and leading to phagocytosis, T- and B-cell activation, and antibody formation.

The spleen's immunologic function is probably less important in the well-developed adult immune system than in the still-developing immune system of the child. Young children who undergo splenectomy may develop overwhelming, often fatal, infections with encapsulated organisms such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. This can also be a rare complication of splenectomy in adults. The loss of the marginal zone can be especially important in this regard.[28]

The red pulp cords of the spleen act as a reservoir for platelets, sequestering approximately one-third of the circulating platelet mass. Massive enlargement of the spleen (splenomegaly) can result in a pooling of 80–90% of the platelets, producing peripheral blood thrombocytopenia. Removal of the spleen results in a transient thrombocytosis with a return to normal platelet concentrations in about 10 days.

Hypersplenism

In a number of conditions, the spleen can become enlarged and, through exaggeration of its normal activities of filtering and phagocytosing, cause anemia, leukopenia, thrombocytopenia, or combinations of cytopenias. A diagnosis of hypersplenism is made when three conditions are met: (1) the presence of anemia, leukopenia, or thrombocytopenia in the peripheral blood, (2) the existence of a cellular or hyperplastic bone marrow corresponding to the peripheral blood cytopenias, and (3) the occurrence of splenomegaly. The correction of cytopenias following splenectomy confirms the diagnosis.

Hypersplenism has been categorized into two types: primary and secondary. Primary hypersplenism is said to occur when no underlying disease can be identified. The spleen functions abnormally and causes the cytopenia(s). This type of hypersplenism is very rare. Secondary hypersplenism occurs in those cases in which an underlying disorder causes the splenic abnormalities. Secondary hypersplenism has many and varied causes. Hypersplenism can occur secondary to compensatory (or workload) hypertrophy of this organ. Inflammatory and infectious diseases are thought to cause splenomegaly by an increase in the spleen's immune defense functions. For example, an increase in clearing particulate matter can lead to an increase in number of macrophages, and hyperplasia of lymphoid cells can result from prolonged infection. Several blood disorders can cause splenomegaly. In these disorders, intrinsically abnormal blood cells or cells coated with antibody are removed from circulation in large numbers (e.g., hereditary spherocytosis, immune thrombocytopenic purpura; Chapters 17 and 19).

Infiltration of the spleen with additional cells or metabolic byproducts can also cause hypersplenism. Such conditions include disorders in which the macrophages accumulate large quantities of undigestible substances; some of these disorders, such as Gaucher's disease, will be discussed later (Chapter 21). Neoplasms in which the malignant cells occupy much of the splenic volume can cause splenomegaly. If the tumor cells incapacitate the spleen, the peripheral blood will show evidence of hyposplenism (similar to the findings after splenectomy). Congestive splenomegaly can occur following liver cirrhosis with portal hypertension or congestive heart failure when blood that does not flow easily through the liver is rerouted through the spleen.

☑ CHECKPOINT 3-4

Contrast primary and secondary hypersplenism and give an example of a disorder that can cause secondary hypersplenism.

Splenectomy

Splenectomy can relieve the effects of hypersplenism; however, this procedure is not always advisable, especially when the spleen is performing a constructive role such as producing antibody or filtering protozoa or bacteria. Splenectomy appears to be most beneficial in patients with hereditary or acquired conditions in which erythrocytes or platelets are undergoing increased destruction, such as hemolytic disorders or immune thrombocytopenia. The blood cells are still abnormal after splenectomy, but the major site of their destruction is removed. Consequently, the cells have a more normal life span. Splenectomy results in characteristic erythrocyte abnormalities that experienced clinical laboratory professionals can note easily on blood smears. After splenectomy, the erythrocytes often contain inclusions (e.g., Howell Jolly bodies, Pappenheimer bodies), and abnormal shapes can be seen (Chapter 10).

The lifespan of healthy erythrocytes is not increased after splenectomy. Other organs, primarily the liver, assume the culling function. Blood flow through the liver also is slowed by passage through sinusoids, which are lined with specialized macrophages called *Kupffer cells*. These macrophages can perform functions similar to those of phagocytes in the splenic cords and marginal zone. Even when a spleen is present, the liver, because of its larger blood flow, is responsible for removing most of the particulate matter of the blood. The liver, however, is not as effective as the spleen in filtering abnormal erythrocytes, probably because of the relatively rapid flow of blood past hepatic macrophages.

Acquired hyposplenism is a complication of sickle cell anemia. The spleen's acidic, hypoxic, hypoglycemic environment leads to sickling of the erythrocytes in the spleen. This leads to blockage of the blood vessels and infarcts of the surrounding tissue. The tissue damage is progressive and leads to functional splenectomy (also referred to as *autosplenectomy*) (Chapter 13).

CASE STUDY *(continued from page 30)*

Francine was diagnosed as having leukemia.

3. What do you think is the cause of the splenomegaly?
4. Why might the peripheral blood reveal changes associated with hyposplenism when the spleen is enlarged?

LYMPH NODES

The lymphatic system is composed of lymph nodes and lymphatic vessels that drain into the left and right lymphatic ducts. Lymph is formed as a filtrate of blood plasma that escapes into connective tissue. The lymphatic vessels originate as lymphatic capillaries in connective tissue spaces throughout the body, collect the lymph, and carry it toward the lymphatic ducts near the neck where the fluid re-enters the blood. The bean-shaped lymph nodes occur in groups or chains along the larger lymphatic vessels. Fluid from the lymphatic vessels enters the lymph node through afferent lymphatic vessels and exit through efferent lymphatic vessels. Lymph nodes contain an outer *cortex* and an inner *medulla* (Figure 3-7 ■). Fibrous trabeculae extend inward from the capsule to form irregular compartments within the cortex. The cortex contains B-cell follicles surrounded by T lymphocytes and macrophages. Similar to the spleen, some follicles contain areas of activated B cells known as *germinal centers*. A stimulated node can have many germinal centers, but a resting node contains follicles with small resting lymphocytes and macrophages. The medulla, which surrounds the efferent lymphatics, consists of cords of B cells, plasma cells, and macrophages that lie between sinusoids.

Lymph nodes act as filters to remove foreign particles from lymph by resident dendritic cells and macrophages. In addition, dendritic cells activated in the tissues can travel via the lymphatics to the lymph nodes. Dendritic cells in turn stimulate T and B cells.

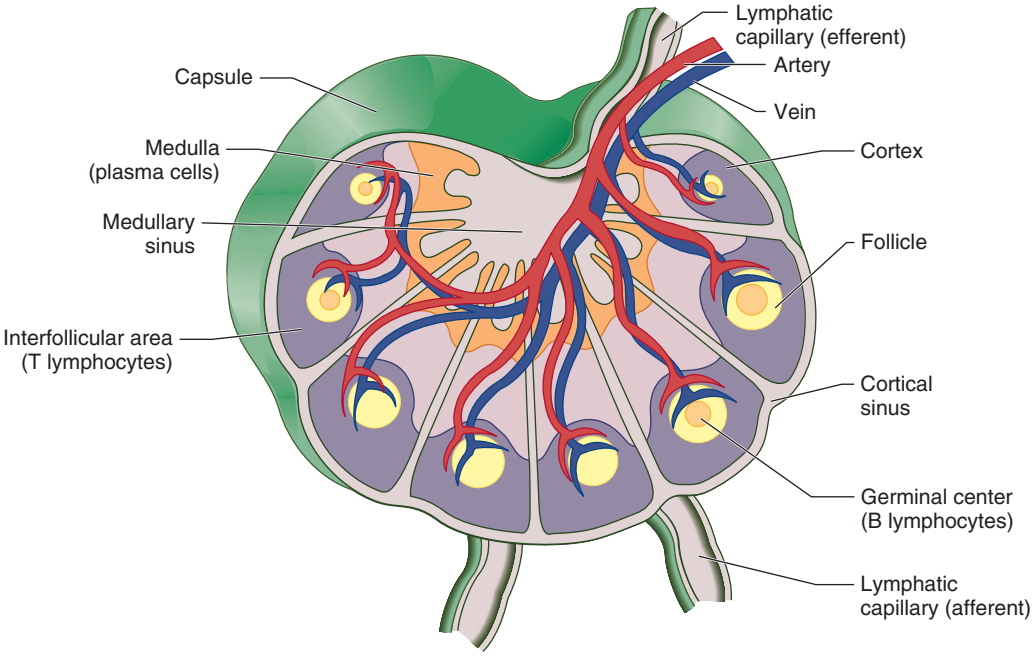


FIGURE 3-7 A schematic drawing of a lymph node. Note the location of T and B lymphocyte populations.

Stimulated B cells move from the germinal centers to the medulla where they reside as plasma cells and secrete antibody. Thus, lymph nodes provide immune defense against pathogens in virtually all tissues.

Mucosa-Associated Lymphoid Tissue (MALT)

Collections of loosely organized aggregates of lymphocytes found throughout the body in association with mucosal surfaces are mucosa-associated lymphoid tissue (MALT).[29] Its basic organization is similar to that of lymph nodes in that T- and B-cell–rich areas can be identified but are not as clearly demarcated as in lymph nodes. The medulla is not present as a separate structure, and no fibrous capsule can be identified. In the intestine, some of these aggregates are known as *Peyer's patches*. Tonsils and the appendix also are part of MALT. Its function is to trap antigens that are crossing mucosal surfaces and initiate immune responses rapidly.

Lymphadenopathy

Lymph nodes can become enlarged by expansion of the tissue within the node due to inflammation, prolonged immune response to infectious agents, or malignant transformation of lymphocytes or macrophages (lymphadenopathy). Alternatively, lymph node enlargement can occur because of metastatic tumors that originate in extranodal ites.

CASE STUDY *(continued from page 33)*

Francine had lymphadenopathy. The leukemia was diagnosed as a leukemia of lymphocytic cells.

5. What might explain the lymphadenopathy?

Summary

Hematopoiesis occurs in several different locations during human development. The major locations include the yolk sac, aorta-gonad-mesonephros (AGM) region, liver, bone marrow, and thymus. Further differentiation of lymphocytes also occurs in the spleen and lymph nodes. In the adult, the bone marrow is the location of hematopoietic stem cells and thus is ultimately responsible for initiating all hematopoiesis. The bone marrow stroma (supporting tissue) provides a microenvironment for proliferation and differentiation of hematopoietic cells (red marrow). The stroma consists of macrophages, reticular cells, and adipocytes. The adipocytes form the yellow marrow and mechanically control the volume of hematopoietic tissue. Myeloid cells, platelets, and erythrocytes essentially complete their differentiation in the bone marrow. T cells finish most of their differentiation in the thymus and secondary lymphoid tissues. B cells are able to respond to antigens by the time

they leave the bone marrow but differentiate further into antibody-secreting plasma cells in the spleen and lymph nodes. The spleen removes senescent or abnormal erythrocytes and particulate matter from erythrocytes. The spleen can become enlarged and through exaggeration of its normal functions cause cytopenias (hypersplenism). Lymph nodes contribute to immune defense by initiating immune responses to foreign particles found in lymph. Lymph nodes can become enlarged due to an immune response to infectious agents or malignant tumor (lymphadenopathy).

Review Questions

Level I

1. B cells develop or differentiate in all of the following tissues *except*: (Objective 2)
   A. thymus
   B. bone marrow
   C. spleen
   D. lymph nodes
2. Lack of a spleen results in: (Objective 4)
   A. younger circulating erythrocytes
   B. granular inclusions in erythrocytes
   C. pitting of erythrocytes
   D. spherocytes
3. Peyer's patches are structurally most closely related to the: (Objective 3)
   A. lymph node
   B. spleen
   C. thymus
   D. liver
4. All of the following are functions of bone marrow stroma *except* that it: (Objective 4)
   A. controls the volume of marrow available for hematopoiesis
   B. provides structural support for marrow elements
   C. secretes growth factors for hematopoiesis
   D. provides an exit route from marrow for mature blood cells
5. Which site of early hematopoiesis is extraembryonic? (Objective 1)
   A. yolk sac
   B. liver
   C. AGM
   D. spleen

Level II

1. A common site of adult extramedullary hematopoiesis is the: (Objective 3)
   A. liver
   B. thymus
   C. lymph node
   D. yolk sac
2. A patient has infectious mononucleosis. His cervical lymph nodes are enlarged. This is most likely due to: (Objective 2)
   A. an immune response to an infectious agent
   B. a malignant tumor
   C. extramedullary hematopoiesis
   D. presence of macrophages containing undigestible substances
3. Gower I, Gower 2, and Portland hemoglobins in erythroblasts characterize: (Objective 5)
   A. erythropoiesis in the spleen
   B. erythropoiesis in the bone marrow
   C. definitive erythropoiesis
   D. primitive erythropoiesis
4. Extramedullary hematopoiesis in the adult is often accompanied by: (Objectives 1, 2, 3)
   A. splenomegaly
   B. liver atrophy
   C. leukocytosis
   D. hyposplenism
5. Culling and pitting of erythrocytes in the circulation takes place in the: (Objective 4)
   A. germinal centers of lymph nodes
   B. cords of red and white pulp and marginal zone of the spleen
   C. cortex of the thymus
   D. sinuses of the bone marrow

6. Primitive erythropoiesis: (Objective 5)
   A. occurs primarily in the liver
   B. originates from a self-renewing hematopoietic stem cell
   C. utilizes the same hemoglobin genes as adults
   D. involves the formation of blood islands

7. Erythroblastic islands are composed of erythrocyte precursors and: (Objective 4)
   A. megakaryocytes
   B. lymphocytes
   C. macrophages
   D. adipocytes

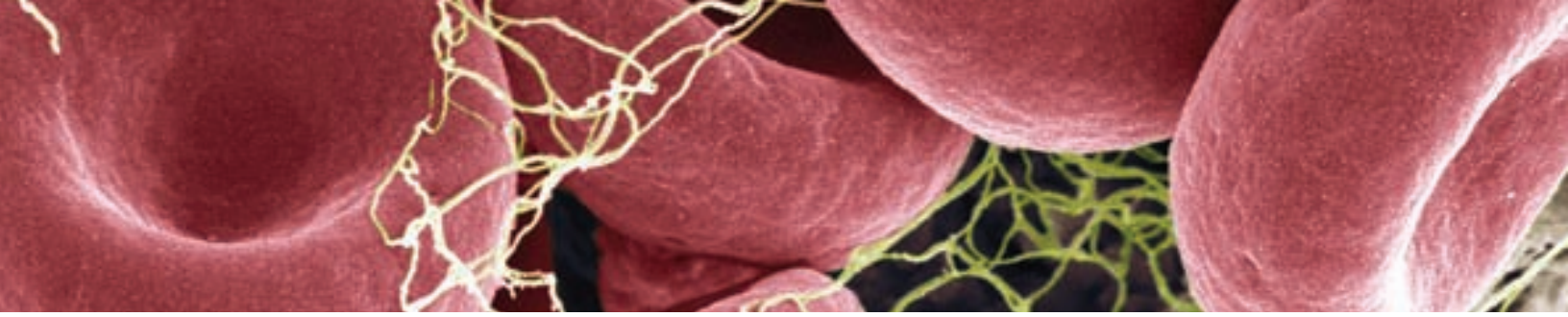
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Palis J, Yoder MC. Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Exp Hematol*. 2001;29:927–36.
2. Gordon S, Fraser I, Nath D et al. Macrophages in tissues and in vitro. *Curr Opin Immunol*. 1992;4:25–32.
3. Marcos MA, Godin I, Cumano A et al. Developmental events from hemopoietic stem cells to B-cell populations and Ig repertoires. *Immunol Rev*. 1994;137:155–71.
4. Ottersbach K, Smith A, Wood A et al. Ontogeny of haematopoiesis: recent advances and open questions. *Br J Haematol*. 2009;148:343–55.
5. Pardanaud L, Luton D, Prigent M et al. Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development*. 1996;122:1363–71.
6. Farace MG, Brown BA, Raschella G et al. The mouse beta h1 gene codes for the z chain of embryonic hemoglobin. *J Biol Chem*. 1984;259:7123–28.
7. Chang Y, Paige CJ, Wu GE. Enumeration and characterization of DJH structures in mouse fetal liver. *EMBO J*. 1992;11:1891–99.
8. Elliott JF, Rock EP, Patten PA et al. The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. *Nature*. 1988;331:627–31.
9. Iversen PO. Blood flow to the haemopoietic bone marrow. *Acta Physiol Scand*. 1997;159:269–76.
10. Afran AM, Broome CS, Nicholls SE et al. Bone marrow innervation regulates cellular retention in the murine hematopoietic system. *Br J Haematol* 1997; 98:569–77.
11. Mayani H, Guilbert LJ, Janowska-Wieczorek A. Biology of the hemopoietic microenvironment. *Eur J Haematol*. 1992;49:225–33.
12. Sugiyama T, Kohara H et al. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 2006 25;977–88.
13. Muruganadan S, Roman AA, Sinal CJ. Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cell Mol Life Sci*. 2009;66:236–53.
14. Rickard DJ, Subramaniam M, Spelsberg TC. Molecular and cellular mechanisms of estrogen action on the skeleton. *J Cell Biochem*. 1999;Suppl 32–33:123–32.
15. Moriyama Y, Fisher JW. Effects of testosterone and erythropoietin on erythroid colony formation in human bone marrow cultures. *Blood*. 1975;45:665–70.
16. Pittenger MF, Mackay Am, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; 284:143–47.
17. Bianco P, Sacchetti B, Riminucci M. Osteoprogenitors and the hematopoietic microenvironment. *Best Pract Res Clin Haematol*. 2011;24:37–47.
18. Sadahira Y, Mori M. Role of the macrophage in erythropoiesis. *Pathol Int*. 1999;49(10):841–48.
19. Lichtman MA, Chamberlain JK, Simon W et al. Parasinusoidal location of megakaryocytes in marrow: a determinant of platelet release. *Am J Hematol*. 1978;4:303–12.
20. Lichtman MA, Packman CH, Costine LS. Molecular and cellular traffic across the marrow sinus wall. In: Tavassol M, ed. *Blood cell formation: The Role of the Hematopoietic Microenvironment*. Clifton, NJ: Humana Press; 1989.
21. Yong, K. Granulocyte colony-stimulating factor (G-CSF) increases neutrophil migration across vascular endothelium independent of an effect on adhesion: comparison with granulocyte macrophage colony stimulating factor (GM-CSF). *Br J Haematol*. 1996;94:40–47.
22. Laurence ADJ. Location, movement and survival: the role of chemokines in haematopoiesis and malignancy. *Br J Haematol*. 2005;132:255–67.
23. Shortman K, Egerton M, Spangrude GJ et al. The generation and fate of thymocytes. *Semin Immunol*. 1990;2:3–12.
24. Zoller AL, Kersh GJ. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of beta-selected thymocytes. *J Immunol*. 2006;176:7371–78.
25. Garcia-Suarez O, Perez-Perez M, Germana A et al. Involvement of growth factors in thymic involution. *Microsc Res Tech*. 2003;62:514–23.
26. Corbeaux T, Hess I, Swann B et al. Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage. *Proc Natl Acad Sci USA* 2010;107:16613–18.
27. Douek DC, Koup RA. Evidence for thymic function in the elderly.*Vaccine*. 2000;18(16):1638–41.
28. Kraal G. Cells in the marginal zone of the spleen. *Int Rev Cytol*. 1992;132:31–74.
29. MacDonald TT. The mucosal immune system. *Parasite Immunol*. 2003;25:235–46.



4 Hematopoiesis

J. LYNNE WILLIAMS, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe the basic concepts of cell differentiation and maturation.
2. Compare and contrast the categories of hematopoietic precursor cells: hematopoietic stem cells, hematopoietic progenitor cells, and maturing cells, including proliferation and differentiation potential, morphology, and population size.
3. Describe the hierarchy of hematopoietic precursor cells and the relationships of the various blood cell lineages to each other (including the concept of colony-forming unit [CFU]).
4. List the general characteristics of growth factors and identify the major examples of early acting (multilineage), later acting (lineage restricted), and indirect acting growth factors.
5. Compare and contrast paracrine, autocrine, and juxtacrine regulation.
6. List examples of negative regulators of hematopoiesis.
7. Define hematopoietic microenvironment.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Compare and contrast the phenotypic characteristics differentiating the hematopoietic stem cells and progenitor cells.
2. Identify the key cytokines required for lineage-specific regulation.
3. Describe the structure and role of growth factor receptors.
4. Summarize the concept of signal transduction pathways.
5. Explain the roles of transcription factors in the regulation of hematopoiesis and differentiation.
6. Outline current clinical uses of cytokines.
7. Identify and describe the cellular and extracellular components of the hematopoietic microenvironment.
8. List and explain the proposed mechanisms used to regulate hematopoietic stem/progenitor cell proliferation/differentiation.

Chapter Outline

Key Terms

Autocrine
Commitment
Cytokine
Differentiation
Extracellular matrix (ECM)
Hematopoiesis
Hematopoietic microenvironment (HM)
Hematopoietic progenitor cell
Hematopoietic stem cell
JAK/STAT signaling pathway
Juxtacrine
Maturation
Maturing cell
Paracrine
Stromal cell
Transcription factor (TF)

Background Basics

The information in this chapter will build on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

LEVEL I AND LEVEL II

- Identify the component parts of a cell, including the structure and function of cellular organelles (Chapter 2).
- Describe the *cell cycle* and the molecules that regulate it (Chapter 2).
- Describe apoptosis and the roles of caspases and the Bcl-2 family of proteins in the process (Chapter 2).
- Summarize the structure of the bone marrow, including the concepts of vascular and endosteal compartments (Chapter 3).

OVERVIEW

This chapter begins with an introduction to the concepts of cellular commitment and differentiation in the hematopoietic system. It discusses the characteristics that define the hematopoietic precursor cells and the cytokines that regulate the development of these precursor cells. The structure and function of the cytokine receptors are presented with a summary of the signaling pathways and transcription factors activated by receptor-cytokine binding. Finally, the hematopoietic microenvironment is described and its role in hematopoiesis summarized.

INTRODUCTION

The maintenance of an adequate number of cells to carry out the functions of the organism is referred to as *tissue homeostasis*. It depends on a careful balance between cellular proliferation, cellular differentiation, and cell death (apoptosis). The hematopoietic system presents a challenge when considering the homeostasis of the circulating blood because the majority of circulating cells are postmitotic cells that are relatively short lived. Thus, circulating blood cells are intrinsically incapable of providing their replacements when they reach the end of their life spans. **Hematopoiesis** is the process responsible for the replacement of circulating blood cells; it depends on the proliferation of precursor cells in the bone marrow that still retain mitotic capability. This process is governed by a multitude of cytokines (both stimulating and inhibitory growth factors) and takes place in a specialized microenvironment uniquely suited to regulate the process.

HEMATOPOIESIS

Cell proliferation and programmed cell death (apoptosis) work together to provide an adequate number of cells (Chapter 2). **Differentiation** is responsible for generating the diverse cell populations that provide the specialized functions needed by the organism. Differentiation is defined as the appearance of different properties in cells that were initially equivalent. Because all cells of an organism carry the same genetic information, differentiation (or the appearance of specific characteristics) occurs by the progressive restriction of other potential developmental programs of the cell. **Commitment** is defined as the instance when two cells derived from the same precursor take a separate route of development.[1] Commitment "assigns the program," and the maturation process executes it (**maturation** includes all phenomena that begin with commitment and end when the cell has all its characteristics).[1]

Hematopoiesis, the development of all the different blood cell lineages, has two striking characteristics: the variety of distinct blood cell types produced and the relatively brief life span of the individual cells. The cells circulating in the peripheral blood are mature blood cells and, with the exception of lymphocytes, are generally incapable of mitosis. They also have a limited life span from days for granulocytes and platelets to ~4 months for erythrocytes. As a result, they are described as *terminally differentiated*. This constant death of mature, functional blood cells by apoptosis means that new cells must be produced to replace those that are removed either as a consequence of performing their biologic functions (e.g., platelets in hemostasis, granulocytes in host defense) or through cellular senescence or "old age" (erythrocytes). The replacement of circulating, terminally differentiated cells depends on the function of less differentiated hematopoietic precursor cells that still retain significant proliferative capabilities. These hematopoietic precursor cells, located primarily in the bone marrow in adults, consist of a hierarchy of cells with enormous proliferation potential. They maintain a daily production of approximately 2×10^{11} red blood cells (RBCs), and 1×10^{11} (each) white blood cells (WBCs) and platelets for the individual's lifetime.[2] In addition, acute stress (blood loss or infection) can result in a rapid, efficient, and specific increase in production over baseline of the particular cell lineage needed. For example, acute blood loss results in a specific increased production of erythrocytes while a bacterial infection results in an increased production of phagocytic cells (granulocytes and monocytes).

Hematopoietic Precursor Cells

The pioneering work of Till and McCulloch began to define the hierarchy of hematopoietic precursor cells using in vivo clonal assays.[3] It was not until the development of in vitro clonal assays, however, that the current model of blood cell production began to evolve.[3–7] Hematopoietic precursor cells can be divided into three cellular compartments defined by their relative maturity: **hematopoietic stem cells**, **hematopoietic progenitor cells**, and **maturing cells** (Table 4-1 ★). The

★ **TABLE 4-1** Comparison of Hematopoietic Precursor Cells

Stem Cells	Progenitor Cells	Maturing Cells
~0.5% of total hematopoietic precursor cells	3% of total hematopoietic precursor cells	>95% of total hematopoietic precursor cells
Multilineage differentiation potential	Restricted developmental potential (multipotential → unipotential)	Committed (unipotential) transit population
Quiescent cell population—population size stable	Population amplified by proliferation	Population amplified by proliferation
Population maintained by self-renewal	Transit population without true self-renewal	Proliferative sequence complete before full maturation
Not morphologically recognizable	Not morphologically recognizable	Morphologically recognizable
Measured by functional clonal assays in vivo and in vitro	Measured by clonal assays in vitro	Measured by morphologic analysis; cell counting differentials

nomenclature used to define these various compartments over the past 20 years has lacked uniformity. Although there is general agreement on the designations *stem cells* and *progenitor cells,* various authors have called the third category precursor cells,[8] maturing cells,[9] or morphologically recognizable precursor cells.[10] In this chapter, we use the term *precursor* to include all cells antecedent to the mature cells in each lineage and the term *maturing cells* to include those precursor cells within each lineage that are morphologically identifiable under the microscope.

Stem Cells

All hematopoiesis derives from a pool of undifferentiated cells, hematopoietic stem cells (HSCs), which give rise to all bone marrow cells by the processes of proliferation and differentiation.[11] The stem cell compartment is the smallest of the hematopoietic precursor compartments, constituting only ~0.5% of the total marrow nucleated cells. However, these rare cells are capable of regenerating the entire hematopoietic system. Thus, they are defined as *multipotential precursors* because they maintain the capacity to give rise to *all* lineages of blood cells. The other defining characteristic of stem cells is their high self-renewal capacity (i.e., they can give rise to daughter stem cells that are exact replicas of the parent cell). Despite their responsibility for generating the entire hematopoietic system, at any one time the majority of stem cells are not dividing (<5%); most are withdrawn from the cell cycle or quiescent (G_0 phase of the cell cycle; Chapter 2).[8]

Stem Cell Phenotype

Stem cells are not morphologically recognizable. Primitive stem cells, isolated by fluorescent-activated cell sorting (FACS), are mononuclear cells very similar in appearance to small lymphocytes. Because stem cells are not *morphologically* identifiable, they have been defined *functionally* by their ability to reconstitute both lymphoid and myeloid hematopoiesis when transplanted into a recipient animal. In mice, the existence of the true HSC has been unequivocally demonstrated by occasional successful transplants with single purified stem cells, thus providing direct proof that single cells capable of sustaining lifelong hematopoiesis do exist.[12] There are no accurate quantitative assays for human HSCs. Despite practical and ethical difficulties surrounding an effective in vivo assay for human stem cells, a number of characteristics have been used to define their phenotype. These can be used in cell-separation protocols and result in a relatively high degree of purity of HSCs. The currently proposed phenotype of human HSC is:

$$CD34^{+}Thy\text{-}1^{+}CD49f^{+}CD38^{-}Lin^{-}HLADR^{-}Rh123^{Lo}$$

In addition, HSCs are positive for the receptor for stem cell factor (SCF-R/c-kit, CD117) and the thrombopoietin (TPO) receptor, TPO-R, (Mpl, CD110). CD34, SCF-R, and TPO-R are not found exclusively on HSCs but also on cells that have begun to differentiate. There is no unique surface marker that definitively identifies an HSC.

CD34 is a 110 kDa glycoprotein expressed by human HSCs and early progenitor cells, as well as vascular endothelial cells.[13] Expression of CD34 is lost as cells mature beyond the progenitor cell compartment. Thy-1 (CD90) is a membrane glycoprotein originally discovered as a thymocyte antigen involved in T-lymphocyte adhesion to stromal cells. More recently, it has been recognized as an important marker in conjunction with CD34 for HSC identification.[14,15] CD49f is the integrin $\alpha 6$ subunit polypeptide, important in cell adhesion.[15] CD38 is a 45 kDa glycoprotein considered to be an early myeloid differentiation antigen. Lin^{-} (lineage negative) refers to the absence of known differentiation markers or antigens present on lineage-restricted progenitors (Table 4-2 ★). The HLA-DR antigens are a component of the human major histocompatibility complex antigens. Rhodamine123 is a fluorescent supravital dye that is taken up by cells.[15] HSCs have high levels of pumps capable of effluxing dyes (and drugs). They transport the dye out of the cells and display low-intensity staining for Rho123 ($Rh123^{Lo}$). Thus, the multipotential stem cells capable of long-term hematopoietic reconstitution are found in the population of cells that contain no lineage-specific antigens, CD38, or HLA-DR antigens but express CD34, Thy-1, CD49f, SCF-R, and TPO-R and are largely quiescent.

The Stem Cell Compartment

Not all HSCs are identical; an "age hierarchy" has been described based on the time it takes for transplanted marrow cells to repopulate a lethally irradiated animal and the duration of resultant hematopoiesis. Thus, the terms *long-term repopulating cells (LTRs)* (which are $Rho123^{Lo}$) and *short-term repopulating cells (STRs)* (which are $Rho123^{Hi}$) are used. The STR cells are further along the hematopoietic developmental pathway, are more likely to be proliferating, and have decreased self-renewal potential[2] (Figure 4-1 ■). STR cells cannot sustain hematopoiesis for

★ **TABLE 4-2** Lineage-Specific Markers Used in Purification of HSCs

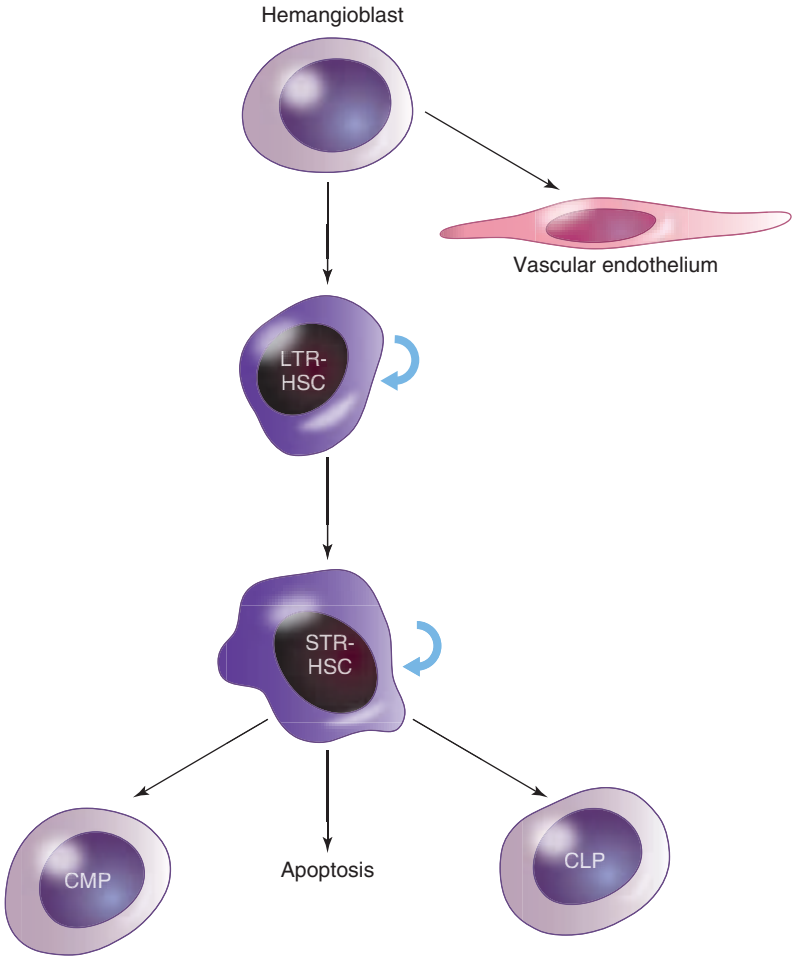
Erythrocytes	Glycophorin A
Megakaryocytes	Glycoprotein (GP) IIb/IIIa
Neutrophils	CD13, CD15, CD33
Monocytes and macrophages	CD11b, CD14
B lymphocytes	CD10, CD19, CD20
T lymphocytes	CD3, CD4, CD5, CD8, CD38, HLA-DR

the recipient animal's lifetime but are more important in blood formation for the first few months after HSC transplantation.[16]

The process of self-renewal is a *nondifferentiating cell division* and ensures that the stem cell population is maintained throughout the individual's lifetime. It is associated with elevated levels of telomerase (which prevents replicative senescence, an irreversible cease in proliferation after a finite number of cell divisions) and Bcl-2 (which prevents apoptosis)[17] (Chapter 2). Humans are estimated to have only ~2×10^4 HSCs.[18] This small group of cells is able to sustain tremendous hematopoietic cell production through the division of only a tiny fraction of its members, keeping the remainder of the stem cells in reserve. The size of the stem cell compartment is relatively stable under homeostatic conditions. In a stem cell compartment that remains stable in size but supplies differentiating cells, a cell must be added to the HSC compartment by proliferation (self-renewal) for each cell that leaves by the process of differentiation. Thus, the stem cell pool must carefully balance the simultaneous processes of expansion (self-renewal) and differentiation. HSCs maintain this balance by a process of asymmetric cell division in which one daughter cell retains all properties of the parent cell (self-renewal), while the other daughter cell undergoes differentiation.[19]

Stem Cell Niches

HSCs reside in unique "stem cell niches" in the bone marrow (BM), where HSCs are retained via adhesion molecules and membrane-bound cytokines. Interactions between HSCs and BM stromal cells



■ **FIGURE 4-1** Derivation and fates of hematopoietic stem cells (HSCs). Hemangioblasts are precursor cells giving rise to both HSCs and vascular endothelium during embryonic development. LTR (long-term repopulating) HSC and STR (short-term repopulating) HSC refer to the length of time these HSC subpopulations take to repopulate depleted hematopoietic tissue and the duration of hematopoiesis arising from each. LTR cells are developmentally more primitive than STR cells. HSCs have three possible fates: self-renewal, commitment to differentiation (becoming common lymphoid progenitors [CLP] or common myeloid progenitors (CMP]), or apoptosis. This cell-fate decision is highly regulated and involves specific transcription factors.

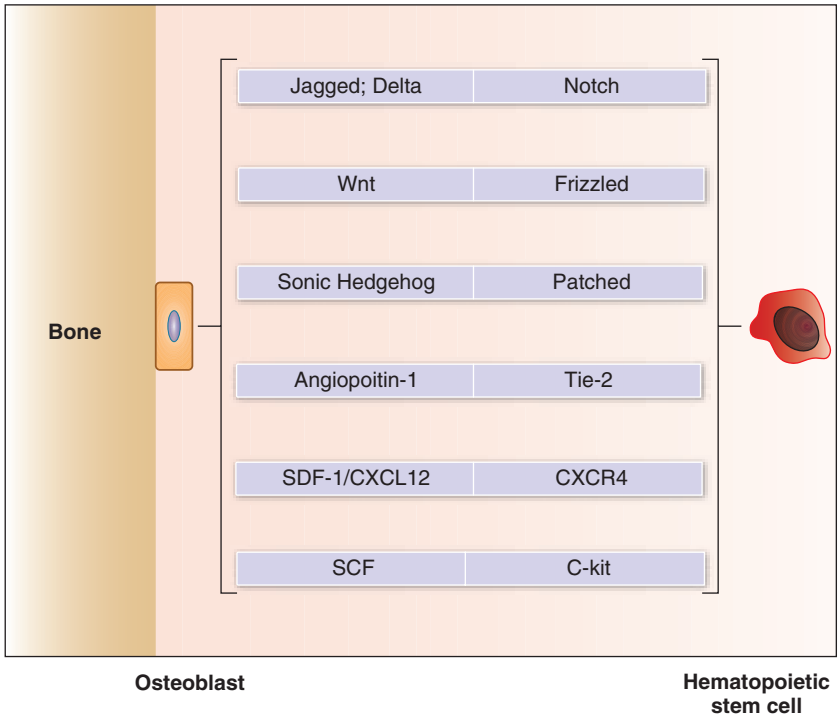
help regulate and balance the processes of self-renewal and differentiation.[20] The niche provides both a physical anchor for the HSCs and factors that regulate HSCs number and function (see later section "Hematopoietic Microenvironment").

There are two important HSC niches.[19] An *osteoblastic niche*, found adjacent to the endosteal surface, supports and maintains HSC quiescence and/or self-renewal.[21] The second is a *vascular niche*, located near the BM sinusoidal endothelial cells, which provides signals for proliferation and differentiation[22] (Chapter 3). Apoptosis, or programmed cell death, can be triggered if the appropriate cytokines or microenvironment is not available to sustain the HSCs (Figure 4-1). Figure 4-1 also depicts the hemangioblast, which is a multipotential precursor capable of producing both HSCs and vascular endothelium.[23]

The regulation of stem cell fate—whether to remain quiescent, self-renew, initiate differentiation, or die—is complex and not fully understood. It is regulated by both cell-intrinsic functions and regulatory signals provided by the HSC niche. Internal cell factors regulating HSCs include SCL (product of stem cell leukemia gene), LMO2 (Lim-only protein 2), and the transcription factors GATA2, AML1, and MYB.[15,17] Abnormal upregulation of many of these factors is seen in acute leukemias and lymphomas (Chapters 26, 27, 42). The osteoblasts in the osteoblastic niche play an important role in regulation of HSC number and function via activation of external ligand-receptor signal pathways between these two cells[15,19] (Figure 4-2 ■). HSC quiescence is maintained through interaction with osteoblasts, molecules in the hematopoietic microenvironment, and cytokines that have an inhibitory effect on hematopoiesis (see "Negative Regulators of Hematopoiesis"). Regulation of the cell cycle determines the HSC choice between quiescence and proliferation. As an example, TGF-β, a negative regulator of hematopoiesis, upregulates the cell-cycle inhibitor p21 (Chapter 2) to help maintain the quiescent status of HSCs.[24]

Progenitor Cells

To meet the cell demands imposed on the hematopoietic system, some stem cells from the HSC compartment initiate differentiation. As HSCs divide, they generate populations of differentiating cells that have an increasingly limited capacity to self-renew and are gradually more restricted in differentiation options.[24] The molecular mechanisms that HSCs utilize to control whether they will self-renew or differentiate upon mitosis remain unresolved. The transition from an HSC to a committed progenitor correlates with the downregulation of HSC-associated genes via gene silencing and the upregulation or activation of lineage-specific genes.[25] Pluripotential stem and progenitor cells simultaneously express low levels of many different genes characteristic of multiple different, discreet lineages (e.g., transcription factors, cytokine receptors).[26] This so-called promiscuous gene expression is characteristic of most multipotent cells. As developing cells downregulate HSC-associated genes, the promiscuous gene expression is reduced; genes of the lineage to which the cell has



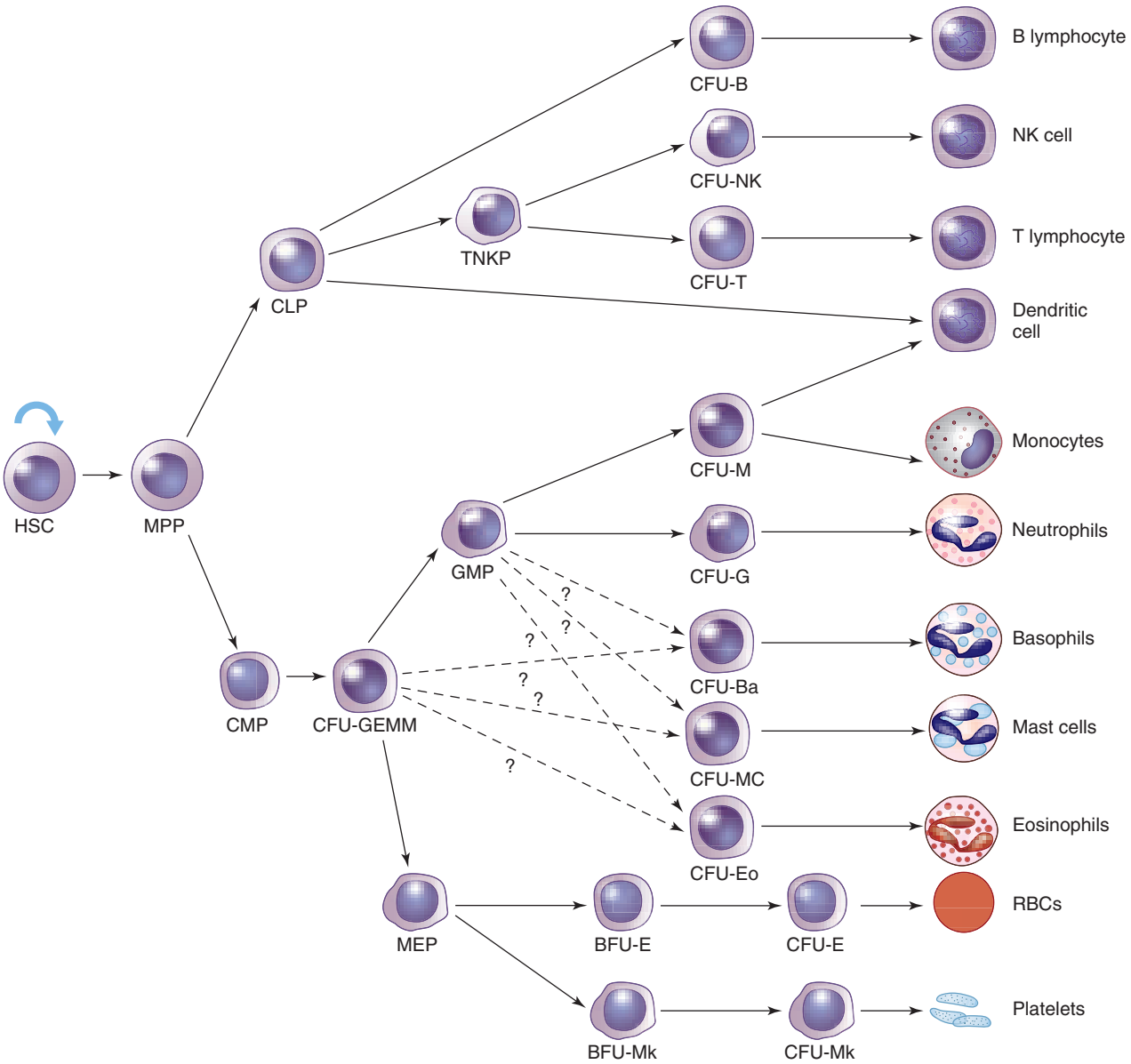
■ **FIGURE 4-2** The osteoblastic HSC niche. The interactions between the endosteal osteoblasts and hematopoietic stem cells are depicted. First three pairs represent ligand (Jagged, Wnt, Sonic Hedgehog)—receptor (Notch, Frizzled, Patched) signaling pathways. The last three pairs represent growth factor (angiopoitin-1, SDF-1, SCF)—receptor (Tie-2, CXCR4, C-kit) interactions. These interactions are thought to determine HSC self-renewal, quiescence, and differentiation.

HSC = hematopoietic stem cell; SCF = stem cell factor; C-kit = stem cell factor receptor.

committed are upregulated while the expression of genes associated with alternate lineages is silenced (epigenetic regulation; Chapter 2). Lineage-specific transcription factors are thought to play essential roles in this process (see section "Transcription Factors").

The Progenitor Cell Compartment

Upon commitment to differentiation, the stem cell enters the next compartment, the hematopoietic progenitor cell (HPC) compartment. Initially, the daughter cells arising from undifferentiated stem cells retain the potential to generate cells of all hematopoietic lineages (multipotential progenitor cells [MPPs]; see Figure 4-3 ■). After additional divisions, however, the progeny of daughter cells progressively lose their ability to generate cells of multiple lineages and gradually become restricted in differentiation potential to a single cell lineage (unilineage or committed progenitor cells). The HPC compartment thus includes all precursor cells developmentally located between HSCs and the morphologically recognizable precursor cells.



■ **FIGURE 4-3** The differentiation of blood cells from a pluripotential stem cell. The pluripotential hematopoietic stem cell (HSC), multipotential progenitor cell (MPP), common myeloid progenitor (CMP), and the colony-forming unit granulocyte, erythrocyte, macrophage, and megakaryocyte (CFU-GEMM) have the potential to differentiate into one of several cell lineages and are therefore multilineage precursor cells. The granulocyte, monocyte progenitor (GMP); megakaryocyte, erythroid progenitor (MEP); and T-lymphocyte, natural killer cell progenitor (TNKP) are bipotential progenitors. The committed (unilineage) progenitors—CFU-G (granulocyte); CFU-M (monocyte); CFU-Eo (eosinophil); CFU-Baso (basophil); BFU (burst-forming unit)-E and CFU-E (erythrocyte); and BFU-Mk and CFU-Mk (megakaryocyte)—differentiate into only one cell lineage. The BFUs are more immature than the CFUs. The mature blood cells are found in the peripheral blood. The common lymphoid progenitor cell (CLP) can differentiate into T or B lymphocytes, natural killer cells, or lymphoid dendritic cells.

The HPC compartment is larger than the HSC compartment, constituting ~3% of the total nucleated hematopoietic cells. HPCs do not possess self-renewal ability; in general, their process of cell division is linked to differentiation. They are, in essence, transit cells said to be on a "suicide" maturation pathway (because full maturation and differentiation result in a *terminally differentiated* cell with a finite life span). Like the HSC, HPCs are not morphologically identifiable but are functionally defined based on the mature progeny that they produce. Both multipotential and unipotential HPCs can be assayed by their ability to form colonies of cells in semisolid media in vitro and are described as *colony-forming units* (CFUs) with the appropriate lineage(s) appended. For example, a CFU-GEMM would be a progenitor cell capable of giving rise to a mixture of all myeloid lineages: **g**ranulocytic, **e**rythrocytic, **m**onocytic, and **m**egakaryocytic; a CFU-Mk would be a unilineage progenitor giving rise exclusively to cells of the megakaryocytic series. HPCs are mitotically more active than stem cells and are capable of expanding the size of the HPC compartment by proliferation in response to increased needs of the body. Thus, the HPC compartment consists of a potentially *amplifying* population of cells as opposed to the stable size of the HSC compartment.

Lineage commitment remains a poorly understood step in hematopoiesis. It is clear, however, that differentiation is accompanied by the increased expression of certain lineage-specific genes and the silencing of genes associated with differentiation along alternate lineages by epigenetic alterations of the chromatin structure (Chapter 2). A number of growth-regulatory glycoproteins or cytokines influence the survival and differentiation of hematopoietic precursor cells (see section "Cytokines and the Control of Hematopoiesis").

Maturing Cells

After a series of amplifying cell divisions, the committed precursor undergoes a further change when the cell takes on the morphologic characteristics of its lineage. Maturing cells constitute the majority of hematopoietic precursor cells; proliferation and amplification boost these cells to >95% of the total precursor cell pool. In general, the capacity to proliferate is lost before full maturation of these cells is complete. They exhibit recognizable nuclear and cytoplasmic morphologic characteristics that can be used to classify their lineage and stage of development. A unique nomenclature is used to categorize these maturing cells morphologically. Generally, the earliest morphologically recognizable cell of each lineage is identified by the suffix *blast* with the lineage indicated (e.g., lymphoblast [lymphocytes], myeloblast [granulocytes], or megakaryoblast [megakaryocytes/platelets]). Additional differentiation stages are indicated by prefixes or qualifying adjectives (e.g., proerythroblast, basophilic erythroblast). A complete discussion of the stages of maturing cells of each lineage can be found in the appropriate chapters (Chapter 5, erythrocytes; Chapter 7, granulocytes; Chapter 8, lymphocytes; Chapter 9, megakaryocytes/platelets).

☑ CHECKPOINT 4-1

Hematopoietic stem cells that have initiated a differentiation program are sometimes described as undergoing death by differentiation. Explain.

Hematopoietic Precursor Cell Model

The head of the hierarchy of hematopoietic cells is the pluripotent *HSC*. These are the cells that have full self-renewal abilities and that give rise to all subsequent hematopoietic precursor cells (Figure 4-3). The progeny of HSCs gradually lose one or more developmental potentials and eventually become committed to a single lineage.

The earliest differentiating daughter cells of the HSC are slightly more restricted in differentiation potential. One group of daughter cells—the *common lymphoid progenitor (CLP) cell*—is a precursor capable of giving rise to all cells of the lymphoid system.[27] The other group—the *common myeloid progenitor (CMP) cell*—is composed of daughter cells restricted to producing cells of the myeloid system (the cell lineages of the bone marrow).[28] Although multipotential, these cells have no self-renewal ability and are ultimately destined to differentiate. The phenotypes for the various levels of HPCs can be seen in Table 4-3 ★.

Following additional differentiation steps (Figure 4-3), the CLP gives rise to T and B lymphocytes, NK (natural killer) cells, and lymphoid dendritic cells; the CMP gives rise to at least six different lines of cellular differentiation, ultimately producing mature neutrophils, monocytes, eosinophils, basophils, erythrocytes, and megakaryocytes/platelets. One of the first precursor cells defined as arising from the CMP was the CFU-GEMM, a cell capable of producing colonies in culture consisting of granulocytic, eosinophilic, erythrocytic, and megakaryocytic elements. However, there are layers of functionally defined cells between the true CMP and the CFU-GEMM. Various authors have assigned names to these intermediate cells, including CFU-Blast,[29] high proliferative potential, colony-forming cell (HPP-CFC),[30] colony-forming unit, day 12 (CFU-D12),[31] and long-term culture initiating cell (LT-CIC).[32]

★ **TABLE 4-3** Phenotype of Hematopoietic Precursor Cells

HSC	$CD34^+$, Thy-1^{Lo}, $CD40f^+$, $CD38^-$, Lin^-, HLA-DR^-, $Rh123^{Lo}$, SCF-R^+, TPO-R^+
CLP	$CD34^+$, Lin^-, $IL7R^+$, Thy-1^-, $SCFR^{lo}$
CMP	$CD34^+$, Lin^-, $IL7R^-$, $SCFR^+$
GMP	$CD34^+$, $SCFR^+$, $Fc\gamma R^{Hi}$, $CD33^+$, $CD13^+$
MEP	$CD34^-$, $SCFR^+$, $Fc\gamma R^{Lo}$, $CD33^-$, $CD13^-$

HSC = hematopoietic stem cell; Lin^- = lineage markers negative; $Rh123^{Lo}$ = negative for supravital dye Rhodamine123; SCF-R = stem cell factor receptor/c-Kit; TPO-R = thrombopoietin receptor/Mpl; CLP = common lymphoid progenitor; IL7-R = IL-7 receptor; CMP = common myeloid progenitor; GMP = granulocyte, monocyte progenitor; MEP = megakaryocytic, erythroid progenitor; FcγR = receptor for Fc component of IgG γ chain.

The sequence of events during the differentiation of a myeloid or lymphoid multipotential progenitor cell to a unilineage, committed progenitor cell is still being resolved. Neutrophils and monocytes are derived from a common committed bipotential progenitor cell, the granulocyte, monocyte progenitor (GMP), which ultimately gives rise to lineage-restricted and morphologically recognizable precursor cells (myeloblasts and monoblasts).[28] Similarly, erythrocytes and megakaryocytes appear to be derived from a common bipotential progenitor cell, the megakaryocyte, erythroid progenitor (MEP),[21,33] and T lymphocytes and natural killer cells share a common precursor, the T lymphocyte, natural killer cell progenitor (TNKP).[34] The developmental pathway for eosinophils and basophils/mast cells remains uncertain. Some authors describe the CFU-Eo and CFU-Ba developing from the GMP[2]; others depict them as deriving from the CFU-GEMM.[35]

Each of the unilineage or committed progenitor cells is named for the cell lineage to which it is committed (e.g., CFU-Mk for megakaryocytes, CFU-E for erythrocytes, CFU-M for monocytes, CFU-G for neutrophils, CFU-Eo for eosinophils, CFU-Ba for basophils). Within some lineages, designated subpopulations of committed progenitor cells are found. Committed erythroid progenitors are designated as erythroid burst-forming units (BFU-E) and erythroid colony forming units (CFU-E) with the BFU-E being the more primitive precursor cell antecedent to the CFU-E. A similar BFU-Mk/CFU-Mk hierarchy has been described for the megakaryocyte lineage.[36] Each committed progenitor cell differentiates into morphologically identifiable precursors of its respective lineage (e.g., CFU-E ⟶ proerythroblast, CFU-G ⟶ myeloblast).

Under normal steady-state physiological conditions, the majority of hematopoietic precursor cells (HSCs and HPCs) are retained in the bone marrow. A small population of HSCs and HPCs, however, can be found circulating in the peripheral blood. The number of circulating HSCs/HPCs can be further increased by the infusion of various cytokines, enabling the collection of "mobilized" peripheral blood HSCs/HPCs for transplantation purposes rather than from a direct bone marrow harvest (Chapter 29).

☑ CHECKPOINT 4-2

Explain the difference in the nomenclature used to label progenitor cells from that used to label maturing cells within the hematopoietic hierarchy of cells.

CYTOKINES AND THE CONTROL OF HEMATOPOIESIS

The regulation of stem cell (HSC) and progenitor cell (HPC) differentiation and expansion is critical because it determines the concentration of the various lineages in the marrow and eventually in the peripheral blood. Specific glycoproteins called *hematopoietic growth factors*, or **cytokines** (Figure 4-4 ■), govern hematopoietic precursor cell survival, self-renewal, proliferation, and differentiation. Growth factor control of hematopoiesis is an extraordinarily complex and highly efficient intercellular molecular communication system that allows coordinated increases in the production and functional activity of appropriate hematopoietic cell types without expansion of irrelevant ones. The first identified growth factors (GFs) were described as *colony-stimulating factors (CSFs)* because they supported the growth of hematopoietic colonies in in vitro cultures. Subsequently, as additional cytokines were discovered, the nomenclature was changed to *interleukins*. When a new cytokine is discovered, the initial description is based on its biologic properties; when the amino acid sequence has been defined, it is assigned an interleukin number. The system has some exceptions and inconsistencies, however. For historic reasons, some cytokines retain their original names (e.g., GM-CSF, G-CSF, M-CSF, EPO, TPO). The initial research into the biologic activities of other cytokines focused on activities outside hematopoietic regulation, and their original names have been retained (e.g., kit-ligand/SCF, Flt3 ligand/FL). At least 37 interleukins have been isolated and characterized to date.

Growth Factor Functions

The growth of hematopoietic precursor cells requires the continuous presence of GFs. If the relevant GFs are withdrawn, the cells die within hours by the process of apoptosis (programmed cell death; Chapter 2). Thus, the first effect of GFs is to promote cell survival by suppressing apoptosis. Second, GFs promote proliferation. Hematopoietic cells are intrinsically incapable of unstimulated cell division. All cell division or proliferation depends on stimulation by appropriate regulatory cytokines. Additionally, GFs control and regulate the process of differentiation, which ultimately produces the mature functional cells from their multipotential progenitor cell precursors (Figure 4-4). Finally, GFs that induce proliferation of precursor cells sometimes have the capacity to enhance the functional activity of the terminally differentiated progeny of these precursor cells.

Characteristics of Growth Factors

Although many different cytokines have been identified as hematopoietic growth factors, some share a number of characteristics (Table 4-4 ★). Most GFs are produced by multiple different cells, including monocytes, macrophages, activated T lymphocytes, fibroblasts, endothelial cells,

★ **TABLE 4-4** Characteristics of Hematopoietic Growth Factors (GFs)

- GFs are produced by stromal cells in the hematopoietic microenvironment.
- Individual GFs have multiple biologic activities (pleiotrophy).
- Many different GFs have similar or identical activities (redundancy).
- By themselves, individual GFs are poor stimulators of colony growth; control of hematopoiesis generally involves the interplay of at least several GFs.
- GFs interact with membrane receptors restricted to cells of appropriate lineage.
- GF requirements change during the differentiation process.
- GFs can affect hematopoiesis directly or indirectly.
- Regulatory cytokines are organized in a complex, interdependent network and exhibit many signal amplification circuits.
- GFs commonly act synergistically with other cytokines.

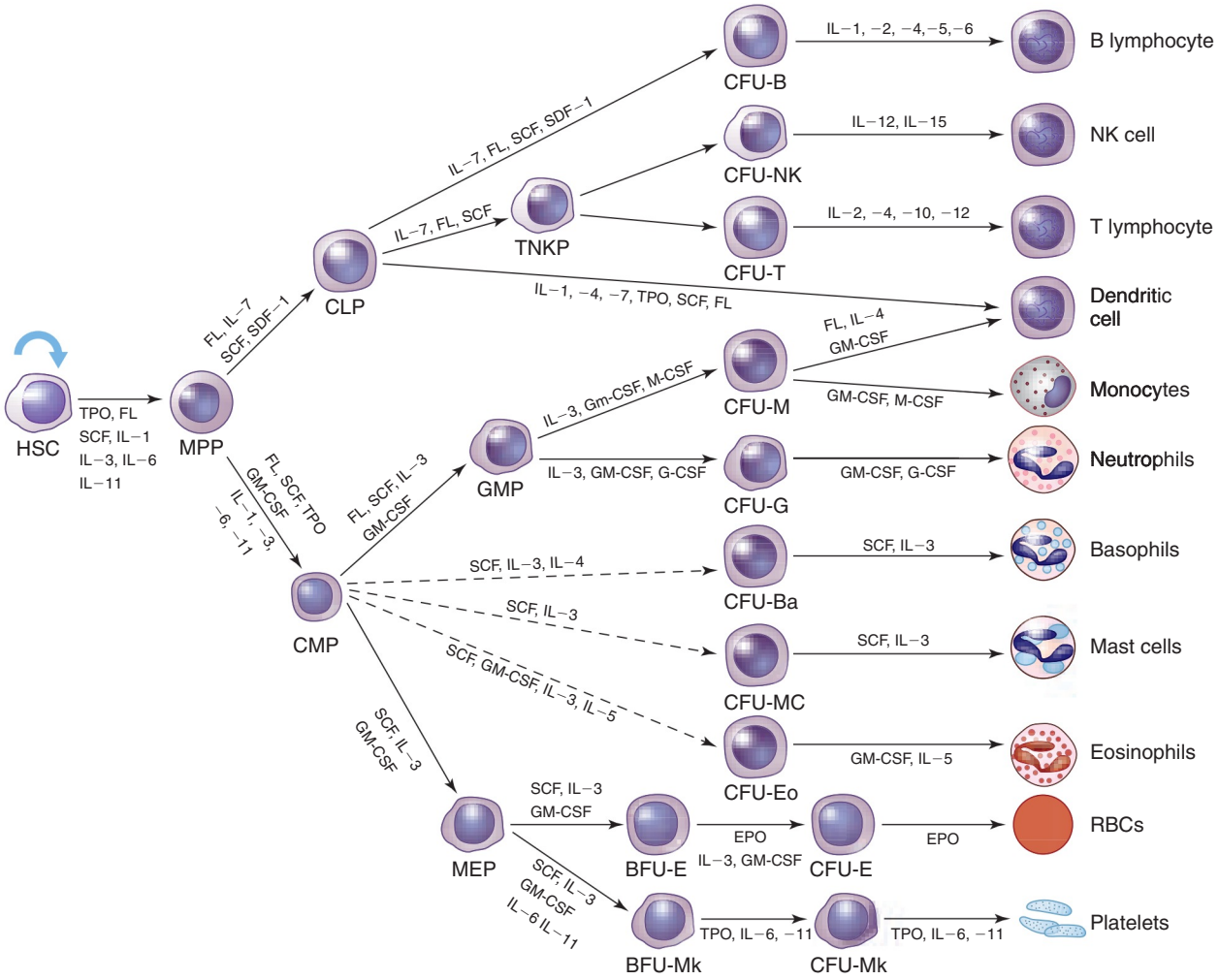


FIGURE 4-4 The pluripotential hematopoietic stem cell (HSC) gives rise to erythrocytes, platelets, monocytes, macrophages, granulocytes, and lymphoid cells. Under stimulation from selective growth factors, stem cell factor (SCF), Flt ligand (FL), and interleukins (IL), the HSC in quiescence (G_0) enters the cell cycle (G_1) and differentiates to the common myeloid progenitor cell (CMP) and, subsequently, to the colony-forming unit-granulocyte, erythroid, macrophage, and megakaryocyte (CFU-GEMM). The CFU-GEMM then differentiates into granulocytes, erythrocytes, monocytes, and megakaryocytes under the influence of specific growth factors, erythropoietin (EPO), thrombopoietin (TPO), granulocyte-monocyte colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and interleukin-5 (IL-5). Different combinations of hematopoietic cytokines regulate the differentiation of HSCs into the common lymphoid progenitor cell (CLP) and subsequently into B and T lymphocytes, natural killer (NK) cells, and lymphoid dendritic cells.

SDF-1 = stromal cell derived factor-1

osteoblasts, and adipocytes (bone marrow stromal cells). Except for erythropoietin (EPO), most GFs are produced by stromal cells in the hematopoietic microenvironment. EPO production is atypical of most lymphohematopoietic GFs in that EPO is produced mainly in the kidney, is released into the peripheral blood, and is carried to the bone marrow where it regulates RBC production. As such, it is the only true hormone (endocrine cytokine); the majority of the other cytokines exert their effects on cells in the local environment where they are produced. Often a single stromal cell source can produce multiple cytokines.

Most GFs are not lineage specific; each GF has multiple functions, and most act on more than one cell type (i.e., they are *pleiotrophic*) (Table 4-5 ★). Cytokines must be bound to surface receptors on their target cells to express their activity. They interact with membrane receptors restricted to target cells of the appropriate responding cell lineage. Because many precursor cells respond to more than one cytokine, they obviously express receptors for multiple GFs. Some GFs influence hematopoiesis directly by binding to receptors on precursor cells and inducing the appropriate response (survival, proliferation, differentiation). Other GFs influence the process indirectly by binding to receptors on accessory cells, which in turn respond by releasing other direct-acting cytokines. Some GFs trigger cell division, and others support survival without inducing proliferation.

Hematopoietic regulatory cytokines interact in a highly ordered, interdependent network creating a complex cell-to-cell

★ **TABLE 4-5** Hematopoietic Growth Factors (GFs)

GF	Mol. Wt.	Chromosome	Source	Major Target Cells/Actions
EPO	34–39,000	7	Kidney (liver)	Erythroid
GM-CSF	18–30,000	5	T cells, BM stromal cells, macrophages	Granulocytes, monos, eos, erythroid, megs, HPCs, DCs
IL-3	14–28,000	5	Activated T cells, mast cells	Myeloid HPCs, mast cells
G-CSF	18,888	17	Monos, macros, BM stromal cells	Granulocytes, early HPCs
M-CSF	70–90,000	1	Monos, macros, BM stromal cells	Monos, macros, osteoclasts
IL-1	17,000	2	Monos, macros, dendritic cells	Monos, ECs, fibroblasts, lymphs, PMNs, early HPCs
IL-2	23,000	4	Activated T_H1 cells	Proliferation and activation of T, B, and NK cells
IL-4	18,000	5	Activated T_H2 cells	Stimulate T_H2 suppress T_H1 B cells, mast cells, basos, fibroblasts
IL-5	50–60,000	5	Activated T_H2 cells, mast cells	Eos, B cells, cytotoxic T cells
IL-6	21–26,000	7	Macros, T_H2 cells, B cells	Early HPCs, B and T cells; megs; myeloma cells
IL-7	17,000	8	Stromal cells (BM and thymus)	Pre-T, pre-B cells, NK cells
IL-8	8,000	4	Monos, macros, ECs	Chemotaxis of granulocytes (chemokine)
IL-9	40,000	5	Activated T_H2 cells	T and B cells, early erythroid cells, mast cells
IL-10	18,000	1	T_H2 cells, monos, macros, activated B cells	B cells, mast cells, T_H2 inhib T_H1 cells
IL-11	24,000	19	BM stromal cells	B cells, megs, early HPC
IL-12	75,000	3,5	Monos, macros, B cells, T cells	T_H1 cells, NK cells
IL-13	18,000	5	T_H2 cells, basos	Isotype switching of B cells; inhib cytotoxic and inflamm functions of monos and macros
IL-14	53–65,000	16	T cells	Activated B cells
IL-15	14–18,000	4	Monos, macros, ECs, fibroblasts	T cells (CTLs), NK cells (LAK), costimulator for B cells
IL-16	16–18,000	15	T cells, eos, epithelial cells	Chemotactic for $CD4^+$ T cells
IL-17	22,000	2	Activated T_H17 cells	Induces cytokine production by stromal cells
IL-18	18,000	7	Macros, keratinocytes	Induces IFN production by T_H1, NK cells
SCF/KL	28–36,000	12	Fibroblasts, ECs, stromal cells	Stem cells, early HPCs, basos and mast cells, melanocytes, germ cells
FL	18,000	19	Stromal cells, monos, macros, T cells	Stem cells, HPCs, B & T precursor cells, DC precursors
TPO	65–85,000	3	Stromal cells, hepatocytes, kidney	Megs, hematopoietic stem cells

T cells, B cells = T or B lymphocytes; NK = natural killer cells; BM = bone marrow; HPCs = hematopoietic progenitor cells; DCs = dendritic cells; ECs = endothelial cells; monos = monocytes; macros = macrophages; basos = basophils; eos = eosinophils; megs = megakaryocytes; PMNs = neutrophils; inhib = inhibits; inflamm = inflammation; activated T cells = T cells activated by antigens, mitogens, or cytokines; CTLs = cytotoxic T lymphocytes; LAK = lymphokine activated killer cells; prolif = proliferation; stim = stimulation.

communication system. Individual GFs by themselves are poor stimulators of colony growth; the control of hematopoiesis generally involves the interplay of at least several GFs. Some GFs act synergistically with other cytokines (*synergism* occurs when the net effect of two or more events is greater than the sum of the individual effects). Many cytokines have overlapping activities (*redundancy*).

The cytokine network often exhibits signal amplification circuits including autocrine, paracrine, and juxtacrine mechanisms of stimulation/amplification (Figure 4-5 ■). **Autocrine** signals are produced by and act on the same cell. **Paracrine** signals are produced by one cell and act on an adjacent cell, typically over short distances. **Juxtacrine** signals represent a specialized type of paracrine signaling in which the cytokine is not secreted by the cell that produced it but remains membrane bound, necessitating direct producer cell–target cell contact to achieve the desired effect. In contrast, endocrine signals (classic hormones) typically act over fairly long distances. The majority of cytokines regulating hematopoiesis exert their effects via paracrine or juxtacrine interactions.

GF requirements change during the differentiation process so that the cytokines/GFs needed by the HSCs and early multipotential HPCs differ from the GF requirements of the later, lineage-restricted progenitors and the maturing precursor cells. These are described as early-acting (multilineage) GFs and later-acting (lineage-restricted) GFs, respectively. GFs and their receptors share a number of structural features, perhaps explaining some of the observed functional redundancies. Most GFs have been cloned and characterized, and recombinant proteins are available; certain of these GFs have been shown to have important clinical applications.

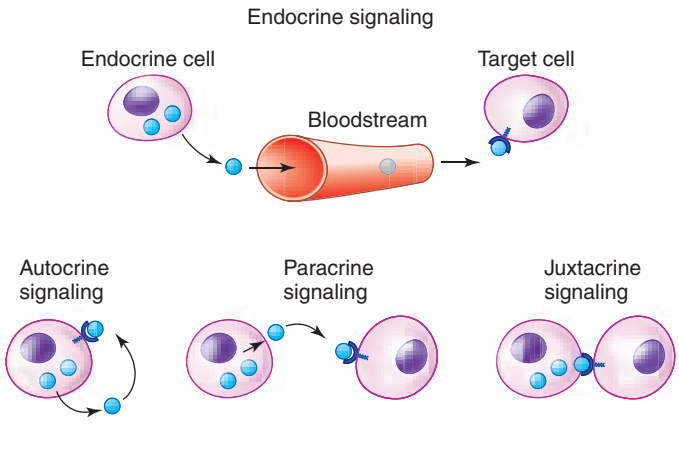


FIGURE 4-5 Mechanisms of cytokine regulation. Autocrine signals are produced by and act on the same cell. Paracrine signals are produced by one cell and act on an adjacent cell, typically over short distances. A juxtacrine signal is a specialized type of paracrine signaling in which the cytokine is not secreted by the producing cell but remains membrane bound, necessitating direct cell–cell contact to achieve the desired effect. In contrast, endocrine signals (classic hormones) typically act over fairly long distances.

Early-Acting (Multilineage) Growth Factors

Several GFs have direct effects on multipotential precursor cells and thus are capable of inducing cell production within several lineages. Early-acting cytokines primarily affect proliferation of these noncommitted progenitor cells. These include SCF, FL, IL-3, GM-CSF, IL-6, and IL-11. Although these factors can initiate proliferation in several cell lineages, additional factors are necessary in many instances for the production of mature cells in these lineages (Figure 4-4).

Stem Cell Factor (SCF) and Flt3 Ligand (FL)

Stem cell factor (SCF) (also known as kit ligand [KL] or mast cell growth factor [MCGF]) suppresses apoptosis of HSCs and promotes the proliferation and differentiation of stem cells, multilineage progenitor cells, and some committed progenitor cells (CFU-GEMM, GMP, CFU-Mk, BFU-E). SCF also promotes the survival, proliferation, and differentiation of mast cell precursors and has functional activity outside the hematopoietic system (melanocyte development and gametogenesis). Flt3 ligand (FL) increases recruitment of primitive HSCs/HPCs into cell cycle and inhibits apoptosis.[37] In contrast to SCF, FL has little effect on unilineage BFU-E/CFU-E, CFU-mast cell, or CFU-Eo but is a potent stimulator of granulocytic/monocytic, B lymphocytic, and dendritic cell proliferation and differentiation. FL and SCF have similar protein structures and share some common characteristics. Both cytokines can be found as either membrane-bound or soluble forms, although the membrane-bound form appears more important physiologically; thus, they operate primarily through juxtacrine interactions.[38] Neither cytokine has independent proliferation-inducing activity, but both act synergistically with IL-3, GM-CSF, G-CSF, and other cytokines to promote early progenitor cell proliferation.

Interleukin 3 and GM-CSF

Interleukin 3 (IL-3) was one of the earliest recognized multipotential growth factors that directly affects multilineage progenitor cells and early committed progenitors such as BFU-E. IL-3 also has indirect actions and can induce the expression of other cytokines. GM-CSF is a multipotential GF that stimulates clonal growth of all lineages except basophils. GM-CSF also activates the functional activity of most mature phagocytes including neutrophils, macrophages, and eosinophils.

Interleukin 6 and Interleukin 11

Interleukin 6 (IL-6) and interleukin 11 (IL-11) are pleiotropic cytokines with overlapping growth stimulatory effects on myeloid and lymphoid cells as well as on primitive multilineage cells.[39,40] Each cytokine rarely acts alone but functions synergistically with IL-3, SCF, and other cytokines in supporting hematopoiesis. Both cytokines have significant effects on megakaryocytopoiesis and platelet production.[41] Both mediate the acute phase response of hepatocytes and are major pyrogens in vivo. IL-6 also stimulates the production of hepcidin, a regulator of iron absorption (Chapter 12).

Later-Acting (Lineage-Restricted) Growth Factors

The growth factors included in this group tend to have a narrower spectrum of influence and function primarily to induce maturation along a specific lineage. Most are *not* lineage specific, however, but instead demonstrate a predominant effect on the committed progenitor cell of a single lineage, inducing differentiation of these more mature cells. These growth factors include granulocyte colony-stimulating factor (G-CSF) (granulocytes), monocyte colony-stimulating factor (M-CSF) (monocytes), erythropoietin (EPO) (erythrocytes), thrombopoietin (TPO) (megakaryocytes and platelets), interleukin-5/IL-5 (eosinophils), and the interleukins important in lymphopoiesis (IL-2, -4, -7, -10, -12, -13, -14, -15).

EPO is the only cytokine to function as a true hormone because it is produced primarily in the kidneys and travels via the circulation to the bone marrow to influence erythrocyte production. It is expressed primarily by hepatocytes in embryonic life and by cells of the kidney (and to a lesser extent, the liver) in adult life. Its release is regulated by the body's oxygen needs and is induced by hypoxia (Chapter 5). EPO stimulates survival, growth, and differentiation of erythroid progenitor cells (with its major effect on CFU-E). It also stimulates proliferation and ribonucleic acid (RNA) and protein synthesis in erythroid-maturing cells. Reticulocytes and mature erythrocytes do not have receptors for EPO and thus are not influenced by this cytokine.

G-CSF, M-CSF, and IL-5 stimulate the proliferation of granulocyte, monocyte/macrophage, and eosinophil progenitor cells, respectively. All three also influence the function of mature cells of their respective lineages, increasing migration, phagocytosis, and metabolic activities and augmenting prolongation of their life spans. M-CSF also regulates the production of osteoclasts, and IL-5 stimulates lymphocyte development.

TPO, also known as *mpl-ligand*, is the major physiologic regulator of megakaryocyte proliferation and platelet production. In vitro, TPO primes mature platelets to respond to aggregation-inducing stimuli and increases the platelet release reaction.[42] TPO also synergizes with a variety of other GFs (SCF, IL-3, FL) to inhibit apoptosis and promote maintenance of HSCs/HPCs.

Indirect-Acting Growth Factors

Some cytokines that regulate hematopoiesis do so indirectly by inducing accessory cells to release direct-acting factors. An example is IL-1, which has no colony-stimulating activity itself. However, when administered in vivo, it induces neutrophilic leukocytosis by promoting the release of other direct-acting cytokines from accessory cells.

Other Stem Cell Regulators

Recently a number of additional factors important in the regulation of HSC function have been described.[15,17] These proteins are important regulators of HSC quiescence, self-renewal, and induction of differentiation within the endosteal HSC niche (Figure 4-2, Table 4-6 ★). There is significant interest in understanding how these factors regulate self-renewal. A more complete understanding of this process is anticipated to allow the development of novel therapeutic approaches for the treatment of hematologic malignancies.

Lineage-Specific Cytokine Regulation

Erythropoiesis

In the erythroid lineage, progenitor cells give rise to two distinct types of erythroid colonies in culture (Chapter 5). A primitive progenitor cell, the BFU-E, is relatively insensitive to EPO and forms large colonies after 14 days in the form of bursts. Production of BFU-E colonies was originally described as being supported by *burst-promoting activity (BPA)*, now known to be IL-3 or GM-CSF. CFU-E colonies grow to maximal size in 7 to 8 days and depend primarily on EPO. The CFU-E are the descendants of BFU-E and subsequently give rise to the first recognizable erythrocyte precursor, the pronormoblast. Other cytokines reported to influence production of red cells include IL-9, IL-11, and SCF. However, EPO is the pivotal factor that functions to prevent apoptosis and induce proliferation/differentiation of committed erythroid progenitor cells and their progeny.

Granulopoiesis and Monopoiesis

Granulocytes and monocytes are derived from a common bipotential progenitor cell, the GMP, derived from CFU-GEMM. Acting synergistically with GM-CSF and/or IL-3, specific GFs for granulocytes and monocytes support the differentiation pathway of each lineage. M-CSF supports monocyte differentiation, and G-CSF induces neutrophilic granulocyte differentiation. Eosinophils and basophils also are derived from the CFU-GEMM under the influence of growth factors IL-5 and IL-3/IL-4, respectively.

Megakaryocytopoiesis/Thrombopoiesis

Platelets are derived from megakaryocytes, which are progeny of the MEP. CFU-Mks are induced to proliferate and differentiate into megakaryocytes by several cytokines. However, the cytokines that induce the greatest increase in platelet production are IL-11 and TPO.

Lymphopoiesis

The growth and development of lymphoid cells from the CLP occurs in multiple anatomic locations including the bone marrow, thymus, lymph nodes, and spleen (Chapter 8). Multiple GFs play a role in T and B lymphocyte growth and development, most of which act synergistically (Figure 4-4).

☑ CHECKPOINT 4-3

Cytokine control of hematopoiesis is said to be characterized by redundancy and pleiotrophy. What does this mean?

Negative Regulators of Hematopoiesis

In addition to the cytokines that function as positive regulators of hematopoiesis, a second group of polypeptides that inhibit cellular proliferation exists (Table 4-7 ★). Either decreasing production of stimulating factors or increasing factors that inhibit cell growth can limit the proliferation of hematopoietic precursor cells. A homeostatic network of counteracting growth inhibitors is secreted in response to GFs, which normally limit cell proliferation after growth stimuli. Some of these negative regulators of hematopoiesis (e.g., transforming growth factor β [TGF-β]) may contribute to the quiescent state of stem cells and early progenitor cells.[26] Several negative regulators have been shown to upregulate cell-cycle inhibitors such as p16 and p21. Others may oppose the actions of positive regulators that act on these same cells. Whether or not precursor cells synthesize DNA and proliferate depends on a balance between these opposing influences.

The interferons and TGF-β suppress hematopoietic progenitor cells by inhibiting proliferation or inducing programmed cell death. Tumor necrosis factor α (TNF-α) directly suppresses colony growth of CFU-GEMM, GMP, and BFU-E, and E-prostaglandins (PGEs) suppress granulopoiesis and monopoiesis by inhibiting GMP, CFU-G, and CFU-M. Acidic ferritins and lactoferrin are products of mature neutrophils

★ **TABLE 4-6** Molecular Regulators of Hematopoietic Stem Cell (HSC) Fate

HSC Receptor Proteins	Osteoblast Ligands	Function
Notch proteins	Jagged, delta proteins	Promote HSC self-renewal; blockade of differentiation
Frizzled proteins (Wnt receptors)	Wnt proteins	Promote HSC self-renewal and expansion
Patched proteins (Shh receptors)	Sonic hedgehog (Shh)	Promote mitosis and initiation of differentiation
Tie-2	Angiopoietin-1	Promote HSC quiescence
CXCR4	SDF1/CXCL12	Promote survival, proliferation of HSC

★ **TABLE 4-7** Negative Regulators of Hematopoiesis

Interferons
TGF-β
TNF-α
PGEs
Acidic isoferritins
Lactoferrin
Di-OH vitamin D_3
T cells and NK cells
SCI (MIP-1α)

that inhibit hematopoiesis via feedback regulation. Di-hydroxyvitamin D_3 (Di-OH Vitamin D3), classically associated with the stimulation of bone formation, also functions to inhibit myelopoiesis. Additionally, cellular components of the immune system, including T cells and NK cells, can function as negative regulators of hematopoiesis.

Stem cell inhibitor (SCI), also known as *macrophage inflammatory protein*-1α (*MIP*-1α), is believed to be a primary negative regulator of stem cell proliferation.[43] It is a local-acting juxtacrine cytokine present in the stromal microenvironment, which functions to maintain quiescent stem cells in the G_0 phase of the cell cycle.

CYTOKINE RECEPTORS, SIGNALING PATHWAYS, AND TRANSCRIPTION FACTORS

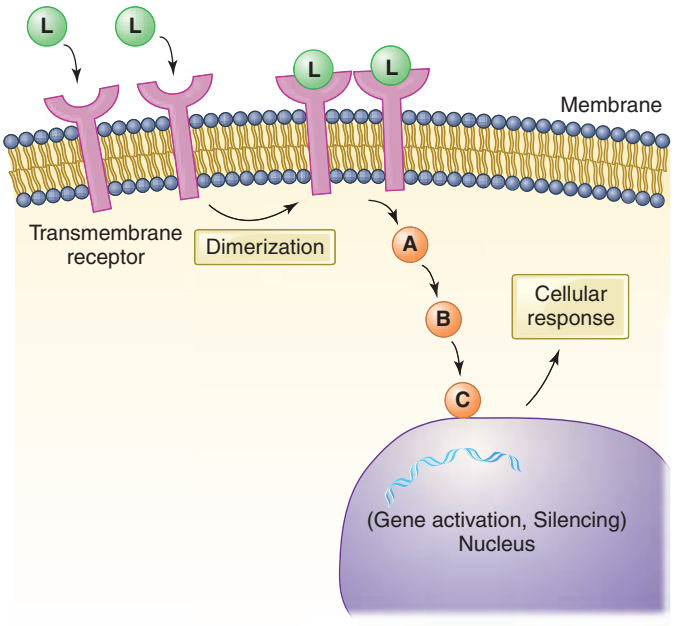
Cytokines must bind to surface receptors on their target cells to express their activity. They interact with membrane receptors restricted to cells of the appropriate lineage. Cells also need a mechanism to transfer signals from extracellular stimuli (cytokines) into appropriate intracellular responses. Binding of a cytokine (ligand) to its specific receptor transduces an intracellular signal through which the particular survival, proliferation, or differentiation responses are initiated. The intracellular portion of the receptor binds to associated intracellular molecules that activate signaling pathways. These signaling molecules translocate to the nucleus, recruit appropriate transcription factors, and activate or silence gene transcription (Figure 4-6 ■). Ultimately, changes in protein synthesis lead to alterations in cell proliferation or other modifications of cellular response induced by the cytokine involved.

Cytokine Receptors

Many receptors for hematopoietic cytokines have been characterized and can be grouped according to certain structural characteristics.[44] Some cytokine receptors, including the receptors for EPO, G-CSF, and TPO, are homodimers (i.e., they consist of two identical subunits). Other receptors are heterodimers or heterotrimers, consisting of different polypeptide subunits (the receptors for most of the other hematopoietic cytokines).

Receptors with Intrinsic Tyrosine Kinase Domains

These receptors, called *receptor tyrosine kinases (RTKs)*, are transmembrane proteins with cytoplasmic regions that contain a tyrosine kinase catalytic site or domain. When GF binds to the receptor,



■ **FIGURE 4-6** A model for the transfer of signals from extracellular stimuli (cytokines) into appropriate intracellular responses. The binding of a cytokine or ligand (L) to its cognate receptor generally induces receptor dimerization, the activation of a cascade of downstream-signaling molecules (A-, B-, C-signal transduction pathways) that converge on the nucleus to induce or repress cytokine-specific genes. The result is an alteration of transcription, RNA processing, translation, or the cellular metabolic machinery.

the receptor chains dimerize, enhancing the catalytic activity of the kinase domain and activating intracellular signaling pathways directly. Included in this group are the receptors for M-CSF, SCF, and FL.

Hematopoietic Growth Factor Receptor Superfamily

The receptors for the majority of hematopoietic GFs do not possess *intrinsic* kinase activity. Cytokine binding and receptor activation induce the docking of cytoplasmic molecules which do have kinase activity, leading to phosphorylation of cellular substrates. All of these receptors are multichain transmembrane proteins that promote signal transduction (i.e., phosphorylation of target cellular proteins) when configured as a heterodimer or homodimer. The receptors for many GF receptors in this large group share peptide subunits with other receptors[16,45] (Web Figure 4-1). The three major subgroups are:

1. IL-3, IL-5, and GM-CSF receptors have unique cytokine-specific α chains but share a common signal-transducing β chain (the βc family).
2. IL-6 and IL-11 similarly have cytokine-specific α chains and share a common signal-transducing β chain called GP130. GP130 is also a subunit of the receptors for several other cytokines, including LIF (leukemia inhibitory factor) and OSM (oncostatin M).
3. The receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 have unique, cytokine-specific α chains and share a common signaling γ chain. IL-2 and IL-15 are actually trimeric structures and share a

common β subunit as well. Inherited abnormalities of the shared γ chain gene are responsible for the X-linked form of severe combined immunodeficiency (SCID) (Chapter 22).

It has been suggested that the functional *redundancy* seen in the cytokine regulation of hematopoiesis (i.e., the fact that multiple GFs often have overlapping activities) can be at least partly explained by the sharing of common receptor signaling subunits. For example, IL-3 and GM-CSF have very similar spectra of biologic activities and share a common β subunit.

Receptor Functional Domains

Most receptors have discrete functional domains in the cytoplasmic region of one or more of the receptor chains. Thus, mutations disrupting a discrete domain of the receptor can disrupt part, but not all, of the functions of that receptor. Kostmann's syndrome (congenital agranulocytosis) is a rare disorder characterized by a profound absolute neutropenia with a maturation arrest of precursor cells at the promyelocyte/myelocyte stage. Erythropoiesis and thrombopoiesis are normal. In some patients, molecular studies have revealed a mutation of the G-CSF receptor that disrupts a terminal *maturation-inducing* domain but leaves intact a subterminal *proliferation-inducing* domain.[45] These patients sustain proliferation of neutrophilic progenitor and early maturing cells, but fail to complete final maturation of cells in this lineage. Similarly, some individuals with previously unexplained primary erythrocytosis (i.e., *not* secondary to smoking, high altitude, or increased EPO levels) have been shown to have a mutation affecting the EPO-receptor (EPO-R).[46] The EPO-R also has been shown to have two separate domains in the cytoplasmic region of the receptor: The domain closest to the membrane constitutes a positive control domain promoting proliferation, and the terminal, negative control domain slows down the intracellular signaling from the receptor. In some patients with familial erythrocytosis, a mutation results in the generation of a truncated receptor that lacks the terminal negative control domain, thus resulting in enhanced responsiveness of target cells (BFU-E and CFU-E) to the growth stimulatory effects of EPO and a (benign) erythrocytosis.

☑ CHECKPOINT 4-4

Individuals with congenital defects of the γ chain of the IL-2 receptor suffer from profound defects of lymphopoiesis far greater than individuals with congenital defects of the α chain of the IL-2 receptor. Why?

Signaling Pathways

As discussed, cells use a variety of "signal transduction pathways" to transfer signals from the cytokine receptor into an appropriate response. These are initiated by a ligand (cytokine) binding to its specific receptor followed by the activation of "downstream signaling molecules," which ultimately converge on the nucleus to modulate transcription, RNA processing, the protein synthetic machinery (translation), the cellular metabolic machinery, or cytoskeletal-dependent functions[47] (Figure 4-6). The signaling cascades that are activated can involve the formation of multiprotein complexes, proteolytic cascades, and/or phosphorylation/dephosphorylation reactions.

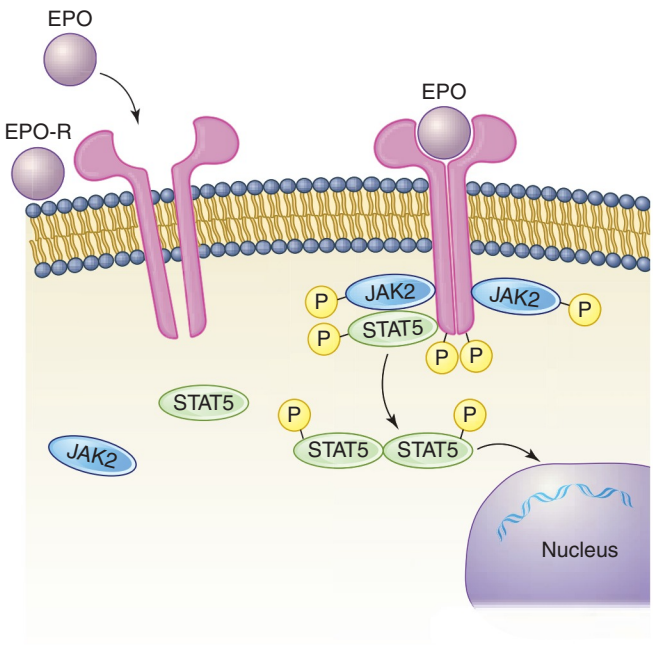
Protein phosphorylation is often an important part of the signaling response from cell-surface receptors involved in hematopoiesis. Receptors that contain intrinsic kinase (or phosphatase) activity are identified by the target amino acid to be phosphorylated or dephosphorylated as receptor tyrosine kinases (RTKs), receptor serine kinases (RSKs), or receptor protein tyrosine phosphatases (PTPs).[47] Ligands activate these receptors by promoting receptor oligomerization and activation of their cytoplasmic kinase domains.

Receptors that do not have intrinsic kinase activity recruit cytoplasmic proteins to their intracellular "tails" and induce the association and assembly of multisubunit protein complexes that generate the enzymatic (phosphorylation) activity. The recruited proteins are termed *protein tyrosine kinases (PTKs)*. Most hematopoietic receptors signal through the Janus family of PTKs, called *JAKs*. Once activated, the JAK kinases recruit molecules that relay the signal, often including members of the STAT family of transcription factors (**S**ignal **T**ransducers and **A**ctivators of **T**ranscription); this pathway is referred to as the **JAK-STAT signaling pathway**. Four different JAK kinases and ~10 different STAT proteins have been identified. Different JAK and STAT proteins are involved in activation of the various hematopoietic lineages. Once STAT proteins are phosphorylated by activated JAK kinases, they dimerize, translocate to the nucleus, bind to cytokine-specific DNA sequences, and activate (or inhibit) specific gene expression[47,48] (Figure 4-7 ■). Abnormalities of the erythrocyte JAK-STAT signaling pathway are the major cause of polycythemia vera (Chapter 24).

Transcription Factors

A cell's phenotype and function are determined by the genes expressed in that cell. Thus, hematopoietic differentiation is regulated by differential gene expression patterns. The growth factors that maintain hematopoiesis are not thought to be "instructive" for the pathway of differentiation but to be "permissive" for cell viability and proliferation.[49] The components that actually establish the patterns of gene expression associated with lineage differentiation are the nuclear **transcription factors (TFs)**. Cell-fate decisions are controlled by the integrated effects of signaling pathways initiated by external cytokines and internal transcription factors.[24]

TFs are DNA binding proteins that interact with the regulatory promoter regions of their target genes. The effect of a particular TF can be either gene expression or gene suppression, depending on the additional molecules (coactivators or corepressors) recruited to the gene promoter region upon TF binding. Different TFs are restricted in their expression to particular lineages and to particular differentiation stages within one or more lineages (Web Table 4-1, Web Figures 4-2 and 4-3a and b). TFs associated with the activation of a particular lineage-specific differentiation program often simultaneously inhibit alternate lineage-specific transcription factors.[50] Interestingly, more than half of the hematopoietic transcription factors identified have been shown to be dysregulated in hematologic malignancies (translocations, point mutations of TF genes)[51] (Chapters 23–28). Thus, the impaired differentiation seen in leukemia is likely due to abnormalities of critical, discrete pathways of transcriptional control.



■ **FIGURE 4-7** Cytokine receptor-JAK-STAT model of signal transduction. Cytokine (e.g., EPO) interaction with its specific receptor (EPO-R) leads to receptor dimerization and activation of JAK kinases associated with the activated receptor. Activated JAK kinases mediate autophosphorylation as well as phosphorylation of the receptor, which then serves as a docking site for signal transducers and activators of transcription (STATs). These STATs are phosphorylated, dissociate from the receptor, dimerize, and translocate to the nucleus where they activate gene transcription.
P = phosphorylated protein

Different TFs are functional at different points in hematopoietic differentiation.[51] Four TFs (or proteins that interact with them) have been identified as important in early embryonic HSCs, and all have been associated with various hematopoietic malignancies. They include SCL/TAL1, AML1/Runx1, MLL, and LMO2. Other TFs involved in either stem cell self-renewal or differentiation include HOX A9, TEL, Bmi1, and Gfi1.

★ **TABLE 4-8** Transcription Factors in Hematopoietic Lineage Differentiation

Hematopoietic Lineage	Transcription Factors
Erythroid/Megakaryocytic	GATA1, FOG1, Gfi-1b, Fli1
Myeloid	PU.1, C/EBPα, C/EBPε, Gfi1, Egr1 and RARα
Lymphoid	PU.1, Ikaros, E2A, EBF, PAX5, Notch1 and GATA3

Although certain TFs are associated with lineage-specific differentiation pathways, many are also expressed, usually at much lower levels, in hematopoietic progenitor cells that are not yet committed to a specific differentiation pathway. This simultaneous expression of TFs for different lineages is thought to explain the progenitor cell's potential for multilineage development.[24] Once a differentiation decision has been made (commitment), upregulation of TFs for one lineage and downregulation or antagonism of the others occur. TFs that specify the various hematopoietic lineages are listed in Table 4-8 ★.[51]

Clinical Use of Hematopoietic Growth Factors

The cloning and characterization of genes encoding the hematopoietic GFs have allowed scientists to produce these cytokines in large quantity using recombinant DNA technology. As a result, GFs can be used in therapeutic regimens for hematopoietic disorders (Table 4-9 ★). Some cytokines approved by the Food and Drug Administration for clinical use include G-CSF and GM-CSF (used to accelerate recovery from granulocytopenia), EPO (for treatment of anemia of various etiologies), IL-11 (for treatment of thrombocytopenia), the interferons (IFNα, IFNβ, and IFNγ used to treat a number of malignant and nonmalignant disorders), and IL-2 (for treatment of metastatic renal cell cancer and melanoma). In vitro studies show that cytokines used in combination often show synergy in terms of their biologic effects. Consequently, the use of combinations of growth factors is being evaluated clinically, often with dramatic results. A more thorough discussion of the biologic therapies currently in clinical use or undergoing clinical evaluations is available.[52]

★ **TABLE 4-9** Clinical Applications of Hematopoietic Growth Factors

Growth Factor	Clinical Applications
EPO	Stimulation of erythropoiesis in a variety of anemias
G-CSF and GM-CSF	Recovery from treatment-induced myelosuppression
IL-3, GM-CSF, EPO	Therapy of myelodysplastic syndromes
IL-2, IFN-α, IFN-β; TGF-β antagonists	Treatment for various malignancies
IL-3, G-CSF, GM-CSF, FL	Priming of bone marrow for donation
IL-1, IL-3, IL-6, IL-11, G-CSF, LIF,	Stimulation of malignant cells to differentiate (variable results in case reports and clinical trials)
IL-1, IL-6	Enhancement of the acute phase response
IL-2, IL-15 (and other lymphocyte-stimulating growth factors).	Enhancement of the immune system
G-CSF, GM-CSF, EPO, IL-11	Stimulation of marrow recovery in BM transplantation
IL-3, G-CSF, GM-CSF	Treatment in bone marrow failure

HEMATOPOIETIC MICROENVIRONMENT

Hematopoiesis is normally confined to certain organs and tissues (Chapter 3). The proliferation and maturation of hematopoietic precursor cells take place within a microenvironment that provides the appropriate milieu for these events.[53,54] Patients undergoing bone marrow transplants receive donor cells by intravenous infusion; the cells "home" to and initiate significant hematopoiesis only in the recipient's bone marrow. No biologically significant hematopoietic activity occurs in nonhematopoietic organs. For successful engraftment, HSCs require an appropriate microenvironment, which presumably has specific properties that make it a unique site for stem cell renewal, growth, and differentiation.

The term **hematopoietic microenvironment (HM)** refers to this localized environment in the hematopoietic organs that is crucial for the development of hematopoietic cells and maintains the hematopoietic system throughout the individual's lifetime. The HM includes cellular elements and extracellular components including matrix proteins and regulatory cytokines (Table 4-10 ★, Figure 4-8 ■). The HM provides homing and adhesive interactions important for the colocalization of stem cells, progenitor cells, and growth-regulatory proteins within the marrow cavity. These are achieved via cell-cell, cell-cytokine, and cell-extracellular matrix interactions.

Components of the Hematopoietic Microenvironment

Cellular Components

The cellular elements of the HM are referred to as hematopoietic **stromal cells** and accessory cells. Stromal cells include adipocytes (fat cells), endothelial cells, fibroblasts, and osteoblasts. Accessory cells include T-lymphocytes, monocytes, and macrophages. The stromal cells' capacity to support hematopoiesis derives from a number of characteristics. These cells are thought to express homing receptors, although the exact mechanisms involved in mediating the homing of hematopoietic cells are unclear. They also produce the various components constituting the extracellular matrix of the HM. Both stromal cells and accessory cells synthesize and secrete soluble growth and differentiation factors and negative regulators as well as a number of membrane-bound cytokines that function as juxtacrine regulators of hematopoiesis (e.g., SCF, FL, SCI). Many of the secreted cytokines bind the extracellular matrix, which concentrates these factors within the HM, keeping them adjacent to the developing hematopoietic precursor cells.

Extracellular Matrix

The stromal cells produce and secrete the **extracellular matrix (ECM)**, which provides the adhesive interactions important for the colocalization of stem cells (HSCs), progenitor cells (HPCs), and the growth-regulatory proteins. The ECM is composed of collagens, glycoproteins, glycosaminoglycans, and cytoadhesion molecules. Variations in the type and relative amounts of these components produce the characteristic properties of ECMs in different tissues. Collagen provides the structural support for the other components. Glycosaminoglycans (heparan-sulfate, chondroitin-sulfate, dermatan-sulfate) play a role in cell–cell interactions, helping to mediate progenitor-cell binding to the stroma. They also serve to bind and localize cytokines in the vicinity of the hematopoietic cells. Cytoadhesion molecules important in hematopoietic cell localization include the β_1 integrins (VLA-4/$\alpha_4\beta_1$, VLA-5/$\alpha_5\beta_1$) found on hematopoietic cells binding to ligands VCAM-1 and fibronectin on marrow stromal cells or in the HM.

Hematopoietic Microenvironment Niches

Within the hematopoietic bone marrow, precursor cells of different lineages and at different stages of differentiation can be found in distinct areas throughout the marrow space. Precursor cells at various stages of differentiation can interact with different ECM components and can be induced by different cytokines to proliferate or differentiate. It has been proposed that specialized stromal cells produce extracellular matrix components and hematopoietic cytokines that are conducive for the commitment and/or differentiation of precursor cells of a specific hematopoietic lineage. These interactions likely contribute to the tight regulation of precursor cell differentiation and proliferation.[54,55]

Adhesion to the Microenvironment

One of the important determinants of the geographic localization of hematopoiesis appears to be the presence of membrane receptors on hematopoietic precursors for stromal cells and ECM proteins. A number of ligand-receptor interactions are important in retaining HSCs in the marrow space. These include SDF-1 (stromal-derived factor 1, also known as *CXCL12*) and its receptor CXCR4, SCF and its receptor SCFR (c-kit), integrins (VLA-4) interacting with their ligands VCAM-1, and hyaluronic acid interacting with its receptor CD44.[20]

Fibronectin is a large, adhesive glycoprotein that binds cells, growth factors, and ECM components. HSCs/HPCs and developing erythroblasts have fibronectin receptors (FnRs) on their surface

★ **TABLE 4-10** Hematopoietic Microenvironment

Cellular (stroma)		Extracellular	
Components	**Function**	**Components**	**Function**
Adipocytes, endothelial cells, fibroblasts, osteoblasts, T cells, macrophages	Expression of homing receptors Production of soluble growth and differentiation factors Production of integral membrane proteins that function as juxtacrine regulators (SCF, FL, SCI) Production of ECM components	Soluble factors (cytokines and growth factors) Extracellular matrix (ECM) Collagen Glycosaminoglycans (heparan-, chondroitin-, dermatan-sulfate) Cytoadhesion molecules	Regulation of hematopoietic stem/progenitor cell differentiation and expansion Structural support Cell-to-cell interactions; localization of growth factors Adhesion of hematopoietic precursors to ECM proteins

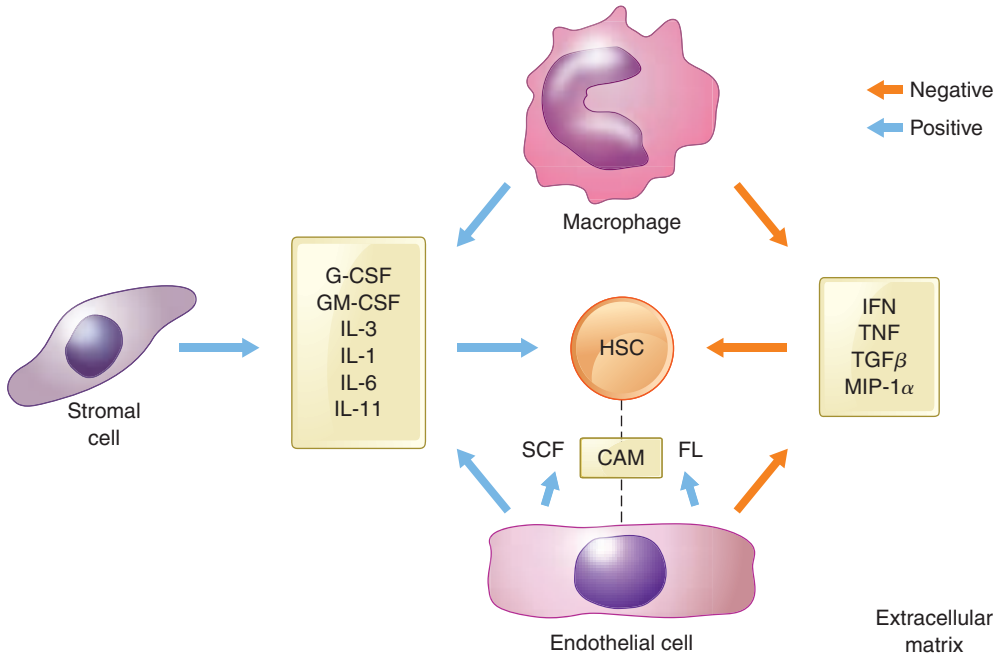


FIGURE 4-8 A model for regulation of hematopoietic precursor cells in the bone marrow microenvironment. The hematopoietic stem cell (HSC) attaches to bone marrow stromal cells via specific receptors and ligands. The HSC is then influenced by both positive and negative regulatory growth factors.

CAM = cell adhesion molecule; SCF = stem cell factor; FL = Flt3-ligand; IFN = interferon; TNF = tumor necrosis factor; TGF- β transforming growth factor β; MIP- 1α = macrophage inflammatory protein- 1α [stem cell inhibitor]; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-monocyte CSF; IL = interleukin

membrane. As developing erythroblasts mature to the reticulocyte stage, they lose their FnRs; loss of attachment via fibronectin facilitates the egress of reticulocytes and erythrocytes from the erythroblastic islands in the bone marrow. Likewise, hemonectin is an adhesive glycoprotein found in the microenvironment that interacts with hemonectin receptors (HnRs) on HSCs, HPCs, and granulocytes and is important for the attachment of these cells to the marrow. Loss of HnRs by developing granulocytes and loss of adhesion to ECM mediates release of mature granulocytes to the circulation. Adhesive interactions between HSCs, HPCs, and the ECM function to help hold the hematopoietic precursor cells in microenvironmental niches, bringing cells into close proximity with growth-regulatory cytokines that are also bound and held by the ECM.[52]

Stem Cell Niche

The quiescent state of stem cells is controlled by their localization in osteoblastic niches that block their responsiveness to differentiation-inducing signals (Figures 4-2 and 4-7, Table 4-6). Stromal cells produce cell-surface–associated (juxtacrine) factors that restrain HSC differentiation. Removal of HSCs from this niche would result in a cascade of differentiation events. A major role of stromal tissue in the regulation of hematopoiesis thus may be to safeguard and ensure stem cell maintenance. Hematopoietic stem cells removed from their marrow environment do not retain their "stemness" for more than a few weeks when cultured in vitro in the absence of stromal cells. Inevitably, they differentiate into progenitor cells and mature cells of the various lineages and thus undergo "death by differentiation."

Recently, it has been recognized that the HSC niche is hypoxic.[54] It has been suggested that the hypoxia of the HSC niche may protect HSCs from oxidative stress. In addition, hypoxia may promote cell-cycle quiescence (an HSC characteristic), which can protect the HSC pool from excess proliferation.

Lymphoid Niches

The bone marrow is the site of B-cell lymphopoiesis (Chapter 8). The less mature developing B cells are located closer to the endosteal surface with the more differentiated cells nearer the sinusoidal endothelial cells.[54] Naïve recirculating B and T cells are also located in the perisinusoidal space in close proximity to dendritic cells. The majority of long-lived memory T cells reside in the bone marrow in close contact with IL-7 secreting stromal cells.[54]

Erythroid Niches

Erythropoiesis occurs in unique anatomical configurations called *erythroblastic islands* (Chapter 5). Some of them are located adjacent to the marrow sinusoids, and others are scattered throughout the bone marrow cavity.

Megakaryocytic Niches

Megakaryocytes tend to localize near the marrow sinusoidal endothelial cells where they are positioned to release platelets into the intravascular sinusoidal space. There is evidence that megakaryocyte localization within a specific vascular microenvironment, mediated by specific cytokines, is necessary for megakaryocyte maturation and platelet production.[54]

Summary

Hematopoiesis is the production of the various types or lineages of blood cells. Mature, terminally differentiated blood cells are derived from mitotically active precursor cells found primarily in the bone marrow in adults. Hematopoietic precursor cells include pluripotential hematopoietic stem cells, hematopoietic progenitor cells (multilineage and unilineage), and maturing (morphologically recognizable) cells.

Hematopoietic growth factors or cytokines (colony-stimulating factors and interleukins) stimulate hematopoietic precursor cells to proliferate and differentiate. Cytokine control of hematopoiesis is characterized by redundancy (more than one cytokine is capable of exerting the same effect on the system) and pleiotrophy (a given cytokine usually exerts more than one biologic effect). These cytokines interact with their target cell by means of unique transmembrane receptors responsible for generating the intracellular signals that govern proliferation and differentiation.

Hematopoiesis takes place in a unique microenvironment in the marrow consisting of stromal cells and extracellular matrix, which plays a vital role in controlling hematopoiesis. It is thought that specialized stromal cells produce extracellular matrix components and hematopoietic cytokines that promote the commitment and/or differentiation of precursor cells of a specific hematopoietic lineage, resulting in lineage-specific niches within the bone marrow.

Review Questions

Level I

1. Self-renewal and pluripotential differentiation potential are characteristics of: (Objective 2)
   A. mature cells
   B. stem cells
   C. progenitor cells
   D. maturing cells

2. Precursor cells that are morphologically recognizable are found in the: (Objective 2)
   A. stem cell compartment
   B. progenitor cell compartment
   C. maturing cell compartment
   D. differentiating cell compartment

3. The MEP gives rise to: (Objective 3)
   A. eosinophils and megakaryocytes
   B. erythrocytes and monocytes
   C. eosinophils and megakaryocytes
   D. erythrocytes and megakaryocytes

4. All hematopoietic cells are derived from the CFU-GEMM *except*: (Objective 3)
   A. lymphocytes
   B. platelets
   C. eosinophils
   D. erythrocytes

5. The following cell that is most sensitive to erythropoietin is: (Objective 4)
   A. reticulocyte
   B. CFU-GEMM
   C. BFU-E
   D. CFU-E

6. All of the following are considered "early acting, multilineage" cytokines *except*: (Objective 4)
   A. IL-5
   B. GM-CSF
   C. SCF
   D. IL-3

7. Pleiotrophy refers to: (Objective 4)
   A. multiple different cells that can produce the same cytokine
   B. a cytokine with multiple biologic activities
   C. multiple cytokines that can induce the same cellular effect
   D. a cytokine that can be produced by multiple different tissues

8. Cytokine regulation in which the cytokine is not secreted by the producing cell but remains membrane bound, necessitating direct cell–cell contact to achieve the desired effect is: (Objective 5)
   A. paracrine
   B. endocrine
   C. juxtacrine
   D. autocrine

9. All of the following are thought to be negative regulators of hematopoiesis *except*: (Objective 6)
   A. TGF-β
   B. SCF
   C. TNF
   D. MIP-1α
10. The hematopoietic microenvironment is composed of: (Objective 7)
   A. hepatocytes and extrahepatic matrix
   B. osteoblasts and osteoclasts
   C. marrow stromal cells and extracellular matrix
   D. hepatocytes and splenic macrophages

Level II

1. Hematopoietic stem cells are characterized by all of the following markers *except*: (Objective 1)
   A. $CD34^+$
   B. Lin^-
   C. HLA-DR^+
   D. Rhodamine123^{Lo}
2. The major molecular marker that differentiates CLP from CMP is: (Objective 1)
   A. IL7-R
   B. FcRγ
   C. CD33
   D. CD13
3. All of the following are important regulators of granulopoiesis *except*: (Objective 2)
   A. GM-CSF
   B. FL
   C. IL-2
   D. IL-3
4. The major cytokine important for eosinophil differentiation is: (Objective 2)
   A. IL-3
   B. IL-5
   C. IL-7
   D. IL-11
5. Which of the following growth factor receptors share a common β chain? (Objective 3)
   A. IL-3 and GM-CSF
   B. TPO and EPO
   C. IL-2 and IL-3
   D. G-CSF and GM-CSF
6. Cytokine receptors that lack an intrinsic kinase domain generally signal: (Objective 4)
   A. through an intrinsic phosphatase domain
   B. by recruiting membrane-embedded kinases
   C. through an intrinsic protease domain
   D. by recruiting cytoplasmic kinases
7. The function of the JAK-STAT pathway in hematopoiesis is to: (Objective 4)
   A. localize cytokines in the hematopoietic microenvironment
   B. generate homing receptors for stem and progenitor cells
   C. produce cytoadhesion molecules to retain precursor cells in the marrow
   D. function as a signal transduction pathway for cytokine-activated receptors
8. The stromal elements of the hematopoietic microenvironment include all of the following *except*: (Objective 7)
   A. B lymphocytes
   B. adipocytes
   C. fibroblasts
   D. osteoblasts
9. Which of the following cytoadhesion molecules plays an important role in retaining erythroid-developing cells in the bone marrow microenvironment? (Objective 7)
   A. hemonectin
   B. fibronectin
   C. thrombospondin
   D. glycosaminoglycans
10. The role of the osteoblastic stem cell "niche" in the bone marrow is thought to be to: (Objective 7)
   A. protect hematopoietic precursor cells from the lytic action of osteoclasts
   B. provide nourishment (oxygen, nutrients) to developing precursor cells
   C. regulate the quiescent state of stem cells blocking differentiation-inducing signals
   D. produce cytoadhesion molecules important for homing to the marrow

Companion Resources

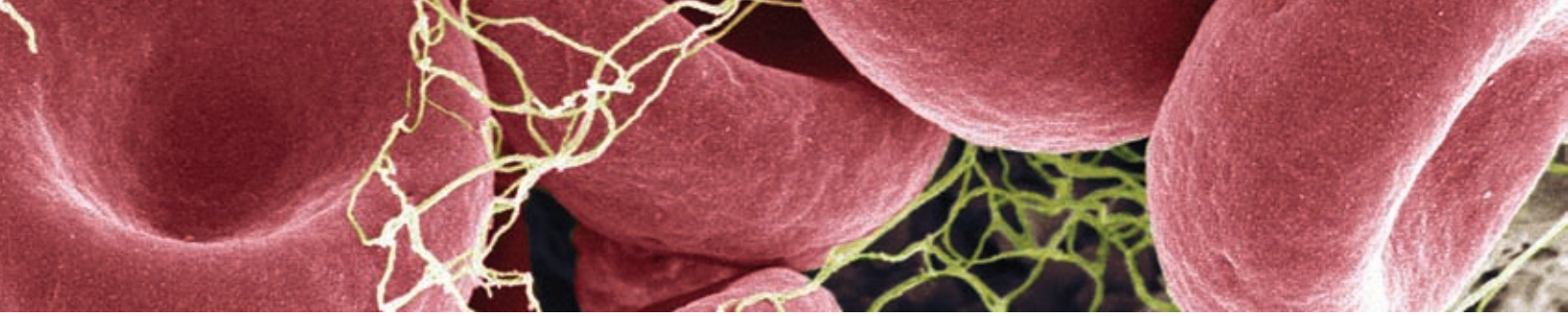
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Bessis M. *Blood smears reinterpreted.* Brecher G, trans. Berlin-Heidelberg-New York: Springer-Verlag; 1977:17.
2. Kaushansky K. Hematopoietic stem cells, progenitors, and cytokines. In: Lichtman MA, Beutler E, Kipps TJ et al., eds. *Williams Hematology*, 7th ed. New York: McGraw-Hill; 2006:201–20.
3. Till JE, McCulloch CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.* 1961;14:213–22.
4. Bradley TR, Metcalf D. The growth of mouse bone marrow cells in vitro. *Aust J Exp Biol Med Sci.* 1966;44:287–99.
5. Silver RK, Erslev AJ. The action of erythropoietin on erythroid cells in vitro. *Scand J Haematol.* 1974;13:338–51.
6. Metcalf D, MacDonald HR, Odartchenko N et al. Growth of mouse megakaryocyte colonies in vitro. *Proc Natl Acad Sci USA.* 1975;72:1744–48.
7. Vainchenker W, Bouguet J, Guichard J et al. Megakaryocyte colony formation from human bone marrow precursors. *Blood.* 1979;54:940–45.
8. Williams DA. Stem cell model of hematopoiesis. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 3rd ed. New York: Churchill Livingstone; 2000:126–38.
9. Lord BI, Testa NG. The hemopoietic system: structure and regulation. In: Testa NG, Gale RP, eds. *Hematopoiesis: Long-Term Effects of Chemotherapy and Radiation.* New York: Marcel Dekker; 1988:1–25.
10. Bagby GC Jr. Hematopoiesis. In: Stamatoyannopoulos G, Nienhuis AW, Majerus PW, Varmus H, eds. *The Molecular Basis of Blood Diseases*, 2nd ed. Philadelphia: W B Saunders; 1994.
11. Prchal JT, Throckmorton DW, Carroll AJ III et al. A common progenitor for human myeloid and lymphoid cells. *Nature.* 1978;274(5671):590–91.
12. Bhatia M, Wang JCY, Kapp U et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci USA.* 1997;94:5320–125.
13. Berenson RJ, Andrews RG, Bensinger WI et al. Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J Clin Invest.* 1988;81:951–55.
14. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell.* 2007;1:635–45.
15. Chute JP. Hematopoietic stem cell biology. In: Hoffman R, Benz EJ, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. New York: Elsevier Saunders; 2013:78–87.
16. Shaheen M, Broxmeyer HE. The humoral regulation of hematopoiesis. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. New York: Churchill Livingstone; 2009:253–75.
17. Schoemans H, Verfaillie C. Cellular biology of hematopoiesis. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. New York: Churchill Livingstone; 2009:200–12.
18. Abkowitz JL, Catlin SN, McCallie MT et al. Evidence that the number of hematopoietic stem cells per animal is conserved in mammals. *Blood.* 2002;100:2665–67.
19. Yoder MC. Overview of stem cell biology. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. New York: Churchill Livingstone; 2009:187–99.
20. Gur-Cohen S, Golan K, Lapid K, et al. Dynamic interactions between hematopoietic stem and progenitor cells and the bone marrow: current biology of stem cell homing and mobilization. In: Hoffman R, Benz EJ, Silberstein LE, et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. New York: Elsevier Saunders; 2013:117–25.
21. Visnjic D, Kalajzic Z, Rowe DW et al. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood.* 2004;103:3258–64.
22. Kiel MJ, Yilmaz OH, Iwashita T et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stemcells. *Cell.* 2005;121:1109–21.
23. Choi K, Kennedy M, Kazarov A et al. A common precursor for hematopoietic and endothelial cells. *Development.* 1998;125:725–32.
24. Zhu J, Emerson SG. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene.* 2002;21:3295–313.
25. Terskikh AV, Miyamoto T, Chang C et al. Gene expression analysis of purified hematopoietic stem cells. *Blood.* 2003;103:94–101.
26. Krause DS. Regulation of hematopoietic stem cell fate. *Oncogene.* 2002;21:3262–69.
27. Galy A, Travis M, Cen D et al. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity.* 1995;3:459.
28. Akashi K, Traver D, Miyamoto T et al. clonogenic common myeloid progenitor that gives rise to all myeloid lineaeges. *Nature.* 2000;404:193.
29. Nakahata T, Ogawa M. Identification in culture of a new class of hemopoietic colony forming units with extensive ability to self-renew and generate multipotential colonies. *Proc Natl Acad Sci USA.* 1982;79:3843–47.
30. Bradley TR, Hodgson GS. Detection of primitive macrophage progenitor cells in mouse bone marrow. *Blood.* 1979;54:1446–50.
31. Magli MC, Iscove NN, Odartchenko V. Transient nature of early haematopoietic spleen colonies. *Nature.* 1982;295:527–29.
32. Sutherland HJ, Lannsdorp PM, Henkelman DH et al. Functional characterization of individual human haematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci USA.* 1990;87:3584–88.
33. McDonald TP, Sullivan PS. Megakaryocytic and erythrocytic cell lines share a common precursor cell. *Exp Hematol.* 1993;21:1316.
34. Ikawa T, Kawamoto H, Fujimoto S et al. Commitment of common T/Natural killer (NK) progenitors to unipotent T and NK progenitors in the murine fetal thumus revealed by a single progenitor assay. *J Exp Med.* 1999;190:1617.
35. Khanna-Gupta A, Berliner N. Granulocytopoiesis and monocytopoiesis. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 4th ed. New York: Churchill Livingstone; 2005:289–301.
36. Long MW, Gragowski LL, Heffner CH et al. Phorbol diesters stimulate the development of an early murine progenitor cell: The burst forming unit-megakaryocyte. *J Clin Invest.* 1985;76:431–38.
37. Veiby OP, Jacobsen FW, Cui L et al. The Flt3 ligand promotes the survival of primitive hemopoietic progenitor cells with myeloid as well as B lymphoid potential: suppression of apoptosis and counteraction by TNF-alpha and TGF-beta. *J Immunol.* 1996;157:2953–60.
38. Flanagan JG, Chan DC, Leder P. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. *Cell.* 1991;64:1025–35.
39. Kopf M, Ramsay A, Brombacher F et al. Pleiotropic defects of IL-6 deficient mice including early hematopoiesis, T and B cell function, and acute-phase responses. *Ann NY Acad Sci.* 1995;762:308–18.
40. Musashi M, Clark SC, Sudo T et al. Synergistic interactions between interleukin-11 and interleukin-4 in support of proliferation of primitive hematopoietic progenitors of mice. *Blood.* 1991;778:1448–51.
41. Du XX, Neven T, Goldman S, Williams DA. Effects of recombinant human interleukin-11 on hematopoietic reconstitution in transplant mice: acceleration of recovery of peripheral blood neutrophils and platelets. *Blood.* 1993;81:27–34.
42. Toombs CF, Young CH, Glaspy JA et al. Megakaryocyte growth and development factor (MGDF) moderately enhances in-vitro platelet aggregation. *Thromb Res.* 1995;80:23–33.
43. Graham GJ, Wright EG, Hewick R et al. Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. *Nature.* 1990;344:442–44.

44. Shaheen M, Broxmeyer HE. The humoral regulation of hematopoiesis. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 4th ed. New York: Churchill Livingstone; 2005:233–65.
45. Dong F, Hoefsloot LH, Schelen AM et al. Identification of a nonsense mutation in the granulocyte-colony-stimulating factor receptor in severe congenital neutropenia. *Proc Natl Acad Sci USA*. 1994; 91:4480–84.
46. de la Chapelle H, Traskelin AL, Jubonen E. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. *Proc Natl Acad Sci USA*. 1993;90:4495–99.
47. Carpenter CL, Neel BG. Regulation of cellular response. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 4th ed. New York: Churchill Livingstone; 2005:47–59.
48. Rane SG, Reddy EP. JAKs, STATs and Src kinases in hematopoiesis. *Oncogene*. 2002;21:3334–58.
49. Orkin SH. Diversification of haematopoietic stm cells to specific lineages. *Nature Reviews*. 2000;1:57–64.
50. Cantor AB, Orkin SH. Transcriptional regulation of erythropoiesis: An affair involving multiple partners. *Oncogene*. 2002;21:3368–76.
51. Dahl R, Hromas R. Transcription factors in normal and malignant hematopoiesis. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. New York: Churchill Livingstone; 2009:213–30.
52. Rowe JM, Avivi I. Clinical use of hematopoietic growth factors. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. New York: Churchill Livingstone; 2009:907–20.
53. Verfaillie CM. Anatomy and physiology of hematopoiesis. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 3rd ed. New York: Churchill Livingstone; 2000:139–53.
54. Silberstein L, Scadden D. Hematopoietic microenvironment. In: Hoffman R, Benz EJ, Silberstein LE, Heslop HE, Weitz JE, Anastasi J, eds. *Hematology: Basic Principles and Practice*, 6th ed. New York: Elsevier Saunders; 2013:88–96.
55. Quesenberry PF, Crittenden RB, Lowry P et al. In vitro and in vivo studies of stromal niches. *Blood Cells*. 1994;2:97–104.



5 The Erythrocyte

JOEL HUBBARD, PhD
STACEY ROBINSON, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. List and describe the stages of erythrocyte maturation in the marrow from youngest to most mature cells.
2. Explain the maturation process of reticulocytes and the cellular changes that take place.
3. Identify the reference interval for reticulocytes.
4. Explain the function of erythropoietin, and include the origin of production, bone marrow effects, and normal values.
5. Describe the function of the erythrocyte membrane.
6. Name the energy substrate of the erythrocyte.
7. Define and differentiate *intravascular* and *extravascular red cell destruction*.
8. State the average dimensions and life span of the normal erythrocyte.
9. Describe the function of 2,3-BPG and its relationship to the erythrocyte.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Summarize the mechanisms involved in the regulation of erythrocyte production.
2. Describe the structure of the erythrocyte membrane, including general dimensions and features; assess the function of the major membrane components.
3. Explain the mechanisms used by the erythrocyte to regulate permeability to cations, anions, glucose, and water.
4. Compare and contrast three pathways of erythrocyte metabolism and identify key intermediates as well as the relationship of each to erythrocyte survival and longevity.
5. Generalize the metabolic and catabolic changes within the erythrocyte over time that "label" the erythrocyte for removal by the spleen.
6. Predict the effects of increased and decreased erythropoietin levels in the blood.

Chapter Outline

Key Terms

Acanthocyte
BFU-E
CFU-E
Cyanosis
Erythroblast
Erythron
Erythropoiesis
Erythropoietin
Glycolysis
Heinz body
Hypoxic
Integral protein
Normoblast
Peripheral protein
Polychromatophilic erythrocyte
Reticulocyte

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the process of cell differentiation and maturation, regulation, and the function of growth factors; describe cell organelles and their function (Chapters 2, 4).
- Give the functional description of the erythroid marrow (Chapter 3).

Level II

- List and describe the function of specific growth factors important in erythrocyte development (Chapter 4).
- Describe the structure and function of the spleen and bone marrow (Chapter 3).

CASE STUDY

We will address this case study throughout the chapter.

Stephen, a 28-year-old Caucasian male of Italian descent, became progressively ill following a safari vacation to West Africa. The patient arrived at the emergency room (ER) for evaluation following several days of fever, chills, and malaise. The advent of hemoglobinuria prompted the patient to seek emergency aid. A clinical history and physical examination supported a diagnosis of anemia. Because Stephen had recently returned from a malarial endemic area, the physician first suspected malaria although the patient had been on primaquine preventive drug therapy while traveling abroad. Blood smears examined for malaria, however, resulted in a negative diagnosis.

Consider what additional laboratory tests could help identify the cause of Stephen's anemia.

OVERVIEW

This chapter is a study of the erythrocyte. It begins with a description of erythrocyte production and maturation. This is followed by an account of the erythrocyte membrane composition and function, cell metabolism, and kinetics of cell production. The chapter concludes with a description of the destruction of the senescent cell.

INTRODUCTION

The erythrocyte (red blood cell, RBC) was one of the first microscopic elements recognized and described after the discovery of the microscope.[1] RBCs play a vital role in physiology, carrying oxygen from the lungs to the tissues where it is utilized in oxidative metabolism. An insufficient number of RBCs results in a condition called *anemia*, which is characterized by inadequate tissue oxygenation. An excess number of circulating RBCs is called erythrocytosis, a condition that has no adverse effect on pulmonary gas exchange.

ERYTHROPOIESIS AND RED BLOOD CELL MATURATION

Erythron refers to the totality of all stages of erythrocyte development, including precursor cells in the marrow and mature cells in the peripheral blood and vascular areas of specific organs such as the spleen. **Erythropoiesis**, or the production of erythrocytes, is normally an orderly process through which the peripheral concentration of erythrocytes is maintained at a steady state. The life cycle of erythrocytes includes stimulation of lineage commitment and maturation of precursor cells in the marrow by **erythropoietin** (the major cytokine regulating erythropoiesis), a circulating life span for mature cells of approximately 100–120 days, and the destruction of senescent cells by mononuclear phagocytic cells in the liver, spleen, and bone marrow.

Erythroid Progenitor Cells

Red cell production begins with the hematopoietic stem cell (HSC) (Chapter 4). Stem cell differentiation is induced by microenvironmental influences to produce a committed erythroid progenitor cell. The committed (unipotential) erythroid progenitor cell compartment consists of two populations of cells, neither identifiable microscopically but defined by their behavior in cell culture systems: the burst-forming unit-erythroid (**BFU-E**) and colony-forming unit-erythroid (**CFU-E**). The BFU-E progenitor cells proliferate under the influence of what was originally called "burst-promoting activity" (BPA), now known to be the cytokine IL-3 or GM-CSF, released by local microenvironmental stromal cells. BFU-Es have a low concentration of erythropoietin (EPO) receptors and thus are relatively insensitive to EPO except in high concentrations. The BFU-Es are defined as progenitor cells that give rise to a "burst" or multifocal colony of cells in an in vitro colony assay in 10 to 14 days. The colony consists of several hundred to several thousand cells recognizable as hemoglobin-containing RBC precursors. Maturation of the BFU-E gives rise to the CFU-E progenitor cell. An individual CFU-E, which undergoes only a few cell divisions, gives rise to a single, discrete colony of 8 to 60 identifiable cells after 2 to 5 days of culture.[2] CFU-Es have a high concentration of EPO

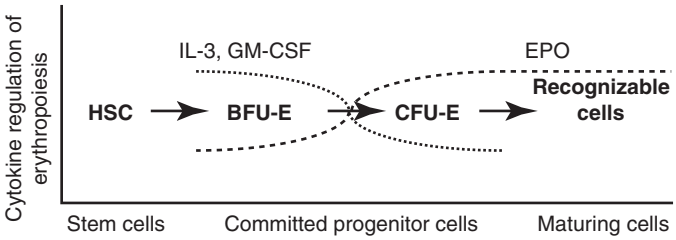


FIGURE 5-1 Erythroid maturation. Erythrocyte development proceeds through three levels of maturation beginning with the multipotential hematopoietic stem cell (HSC), maturing into committed progenitor cells BFU-E and CFU-E, and into morphologically recognizable cells. IL-3 and GM-CSF are the primary cytokines that affect maturation of BFU-E. EPO primarily affects the CFU-E and developing normoblasts.

membrane receptors; hence, they respond to lower EPO concentrations than do BFU-Es. The CFU-E is the immediate precursor of the earliest morphologically recognizable erythroid precursor, the pronormoblast. Figure 5-1 ■ shows the relationship of the various hematopoietic progenitor cells to the cytokines that affect their maturation.

BFU-Es are positive for CD34 ($CD34^+$), a progenitor cell marker, and have a high proliferative potential but a low rate of cycling. As they mature to the CFU-E stage, they lose CD34 expression but begin to express surface proteins characteristic of the erythroid lineage including glycophorin A, Rh antigens, and in a subset of CFU-E, the ABH and Ii antigens.[2]

Additional cytokines shown to have a positive effect on erythrocyte precursor proliferation include stem cell factor (SCF), thrombopoietin, and IL-11; tumor necrosis factor α (TNFα), transforming growth factor-β (TGF β), and interferon-γ(INFγ) have a negative effect on erythropoiesis.

Erythroid-Maturing Cells

Erythroid-maturing cells include those precursor cells in the bone marrow that are morphologically identifiable. Nucleated erythrocyte precursors in the bone marrow are collectively called **erythroblasts**. If the maturation sequence is "normal," the cells are often called **normoblasts**. Young erythrocytes with residual RNA but without a nucleus are referred to as **reticulocytes (polychromatophilic erythrocytes)**. Bone marrow normoblast maturation occurs in an orderly and well-defined sequence under normal conditions encompassing six morphologically defined stages. The process involves a gradual decrease in cell size together with progressive condensation of the nuclear chromatin and eventual expulsion of the pyknotic nucleus. The cytoplasm in the younger normoblasts stains deeply basophilic due to the abundance of RNA (Figure 5-2 ■). As the cell matures, there is an increase in hemoglobin production, which is acidophilic, and the cytoplasm takes on a gray to pink or salmon color (Figures 5-2 and 5-3 ■).

Two terminology systems have been used in the past to describe erythrocyte precursors. One uses the word *normoblast* and the other *rubriblast*. This chapter uses the normoblast terminology; the rubriblast terminology is outdated and rarely used today. The stages in order from most immature to mature cell are BFU-E, CFU-E, pronormoblast, basophilic normoblast, polychromatophilic normoblast, orthochromatic normoblast, reticulocyte, and erythrocyte (Table 5-1 ★).

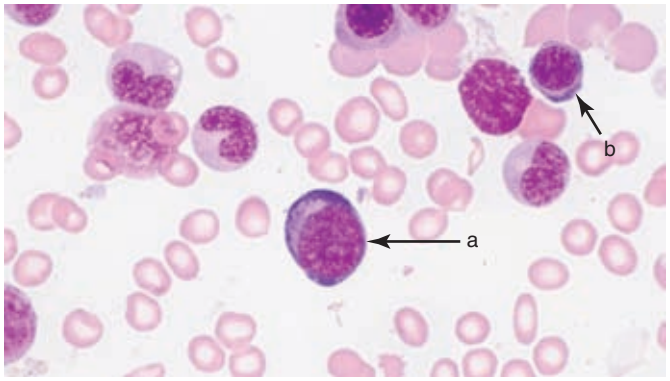


FIGURE 5-2 Pronormoblast in the center (arrow) (a). Note the large N:C ratio, presence of nucleoli, and lacy chromatin. The cytoplasm is deep blue with a light area next to the nucleus. Also note above at about 1 o'clock the polychromatophilic normoblast (arrow) (b). (Bone marrow; Wright-Giemsa stain, 1000× magnification)

The normoblasts generally spend from 5 to 7 days in the proliferating and maturing compartment of the bone marrow. After reaching the reticulocyte stage, there are an additional 2 to 3 days of maturation, the first 1 to 2 days of which are spent in the marrow before the cell is released to the peripheral blood. The mature erythrocyte remains in the circulation for approximately 120 days. This lengthy life span accounts for the relatively small number of erythroid precursors in the marrow in comparison to the large circulating erythrocyte mass.

Erythropoietin (EPO) is the only cytokine important in regulating the final stages of erythroid maturation (the maturing cells). A number of hormones are known to have some erythropoietic effect, however. The most notable is the erythropoietic effect of androgens, which was exploited for the treatment of various anemias before the development of recombinant EPO. Androgens appear to both

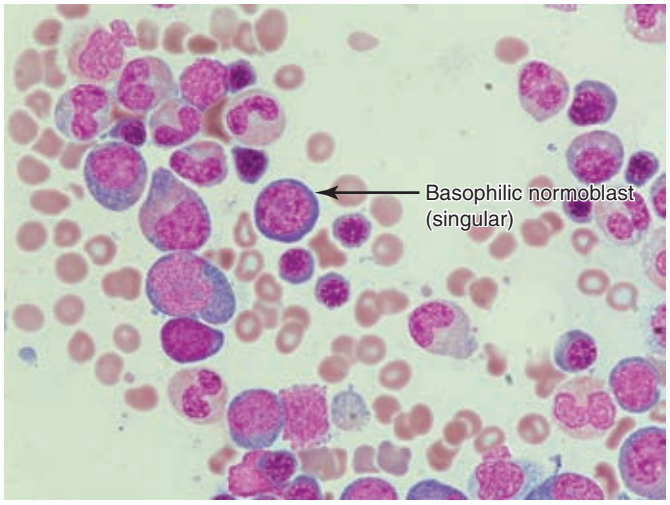
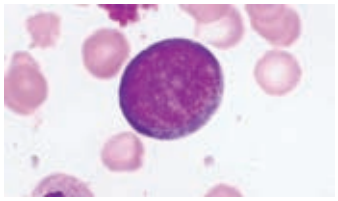
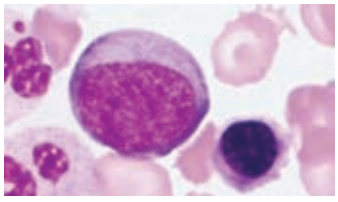
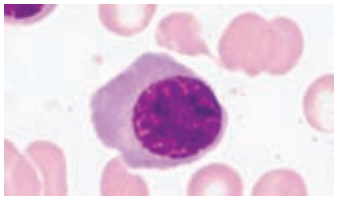
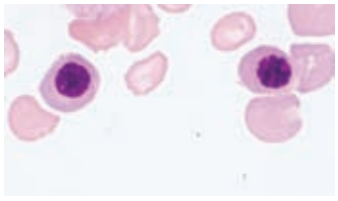
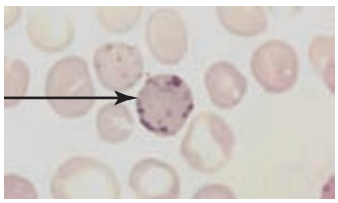
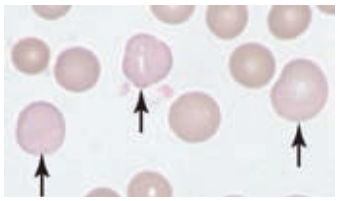
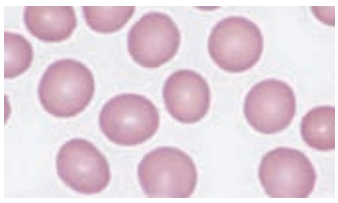


FIGURE 5-3 Developing normoblasts. At left center is a basophilic normoblast; below this are three polychromatophilic normoblasts (bone marrow; Wright-Giemsa stain, 1000× magnification).

★ TABLE 5-1 Morphologic Characteristics of Erythroid Precursors

Cell Type (% of nucleated cells in BM)	Image (Wright stain)	Size	N/C Ratio	Cytoplasmic Characteristics	Nuclear Characteristics
Pronormoblast (1%)		20–25 mcM	High (8:1)	Small to moderate amount of deep blue cytoplasm; pale area next to nucleus may be seen	1–3 faint nucleoli; reddish purple color; homogeneous, lacy chromatin
Basophilic normoblast (1–3%)		16–18 mcM	Moderate (6:1)	Deep blue-purple color; occasionally small patches of pink; irregular cell borders can be present; perinuclear halo can be apparent	Indistinct nucleoli; coarsening chromatin; deep purplish-blue color
Polychromatophilic normoblast (13–30%)		12–15 mcM	Low (4:1)	Abundant; gray-blue color	Chromatin irregular and coarsely clumped, eccentric
Orthochromic normoblast (1–4%)		10–15 mcM	Low (1:2)	Pink-salmon color; can have varying degrees of basophilia	Small; dense; pyknotic; round or bizarre shape; eccentric
Reticulocyte (new methylene blue stain)		7–10 mcM	—	Punctate purplish-blue inclusions	No nucleus
Polychromatophilic erythrocytes				Polychromatophilic (diffusely basophilic)	No nucleus
Mature RBC		7–8 mcM	—	Salmon color	No nucleus

stimulate EPO secretion and directly affect the erythropoietic marrow, which partially explains the difference in hemoglobin concentrations according to sex and age. Other hormones that have varying (although minor) effects on erythropoiesis include thyroid hormones, adrenal cortical hormones, and growth hormone.[3] Anemic patients with hypopituitarism, hypothyroidism, and adrenocortical insufficiency show an increase in erythrocyte concentration when the appropriate deficient hormone is administered. The reduction of EPO in hypothyroidism could partially be the result of the reduced demand for cellular oxygen by metabolically hypoactive tissue.

☑ CHECKPOINT 5-1

What is meant by the term *erythron*?

Characteristics of Cell Maturation

Although the stages of erythrocyte maturation are usually described in a steplike fashion, the actual maturation is a gradual and continuous process (Table 5-1). Some normoblasts might not conform to all criteria for a particular stage, and a judgment must be made when identifying those cells. The more experience one has in examining the blood and bone marrow, the easier it becomes to make these judgments.

Pronormoblast

The earliest morphologically recognizable erythrocyte precursor is the pronormoblast. Each pronormoblast produces between 8 and 32 mature erythrocytes (a total of 3–5 cell divisions during the maturation sequence). This cell is the largest of the normoblast series, from 20–25 mcM (μm) in diameter with a high nuclear-to-cytoplasmic ratio (N:C). The nucleus occupies 80% or more of the cell. This cell is rather rare in the bone marrow.

Cytoplasm

The cytoplasm contains a large number of ribosomes and stains deeply basophilic. A pale area next to the nucleus is sometimes apparent. This represents the Golgi apparatus, which does not take up the dyes of the Romanowsky stain. Small amounts of hemoglobin are present ($<$1% of total cytoplasmic protein) but are not visible by light microscopy.

Nucleus

The nucleus is large, taking up most of the cell volume, and stains bluish-purple. The chromatin is in a fine linear network often described as lacy. The pronormoblast chromatin has a coarser appearance and stains darker than the chromatin of a white cell blast. The nucleus contains from one to three faint nucleoli.

Basophilic Normoblast

This cell is similar to the pronormoblast except that it is usually smaller (16–18 mcM in diameter) and has a slightly decreased N:C ratio. The nucleus occupies approximately three-fourths of the cell volume. Because these cells are actively dividing, it is possible to find a basophilic normoblast (in G_2, prior to mitosis) that is larger than the pronormoblast (in G_1).

Cytoplasm

The cytoplasm is still deeply basophilic, often more so than that of the previous stage due to the increased number of ribosomes. However, in late basophilic normoblasts, the presence of varying amounts of hemoglobin can cause the cell to take on a lighter blue hue or show scattered pink areas. A pale area surrounding the nucleus, the perinuclear halo, is sometimes seen. This halo corresponds to the mitochondria, which also do not stain with Romanowsky stains.

Nucleus

The chromatin is coarser than that of the pronormoblast. The dark violet heterochromatin interspersed with the lighter-staining euchromatin can give the chromatin a wheel-spoke appearance. A few small masses of clumped chromatin can be seen along the rim of the nuclear membrane. Nucleoli usually are not apparent.

Polychromatophilic Normoblast

The cell is about 12–15 mcM in diameter with a decreased N:C ratio due to continued condensation of the nuclear chromatin. This is the last stage capable of mitosis.

Cytoplasm

The most characteristic change in the cell at this stage is the presence of abundant gray-blue cytoplasm. The staining properties of the cytoplasm are due to the synthesis of large amounts of hemoglobin and decreasing numbers of ribosomes. The cell derives its name, polychromatophilic, from this characteristic cytoplasmic feature.

Nucleus

The nuclear chromatin is irregular and coarsely clumped due to increasing aggregation of nuclear heterochromatin.

Orthochromic Normoblast

This cell is about 10–15 mcM in diameter with a low N:C ratio.

Cytoplasm

After the final mitotic division of the erythropoietic precursors, the concentration of hemoglobin increases within the erythroblast. The cytoplasm is predominantly pink or salmon color but retains a tinge of blue due to residual ribosomes.

Nucleus

The nuclear chromatin is heavily condensed. Toward the end of this stage, the nucleus is structureless (pyknotic) or fragmented. The nucleus is often eccentric or even partially extruded. Using phase-contrast microscopy, these cells demonstrate motility with protraction and retraction of cytoplasmic projections.[4] These movements prepare for ejection of the nucleus, which usually occurs while the erythroblast is still part of the erythroblastic island (EI; see section "Erythroblastic Islands"). Proper enucleation requires interaction between erythroblasts and the macrophage of the EI, mediated by a protein called *erythroblast macrophage protein/EMP*.[2] Alternatively, the nucleus can be lost as the cell passes through the wall of a marrow sinus. The expelled nucleus is engulfed by a marrow macrophage.

Reticulocyte

After the nucleus is extruded, the cell is known as a *reticulocyte*. When the nucleus is first extruded, the cell has an irregular lobulated or puckered shape. The cell is remodeled, eliminating excess membrane and gradually acquiring its final biconcave shape while it completes its maturation program.[5]

The reticulocyte has residual RNA and mitochondria in the cytoplasm, which give the young cell a bluish tinge with Romanowsky

stains; thus, the cell is appropriately described as a diffusely basophilic erythrocyte or polychromatophilic erythrocyte. About 80% of the cell's hemoglobin is made during the normoblast stages with the remaining 20% made during the reticulocyte stage. The reticulocyte is slightly larger, 7–10 mcM, than a mature erythrocyte. These cells can be identified in vitro by reaction with supravital stains, new methylene blue N, or brilliant cresyl blue, which cause precipitation of the RNA and mitochondria. This supravital staining method identifies the reticulocytes by the presence of punctate purplish-blue inclusions (Chapter 37). Normally, reticulocytes compose 0.5–2.0% (absolute concentration 25–75 $\times$ 10^9/L) of peripheral blood erythrocytes in a nonanemic adult. The absolute concentration of reticulocytes is calculated by multiplying the percent of reticulocytes by the RBC count. When reticulocytes are increased, an increased number of polychromatophilic erythrocytes (polychromasia) can be seen on the Romanowsky-stained peripheral blood smear.

Reticulocytes can contain small amounts of iron dispersed throughout the cytoplasm, which can be identified with Perl's Prussian blue stain. Erythrocytes with identified iron are called *siderocytes*. Nucleated RBC precursors that stain with Prussian blue are called *sideroblasts*. The spleen is responsible for removal of these iron-containing granules, and the normal mature circulating cell is devoid of granular inclusions.

☑ CHECKPOINT 5-2

What is the first stage of red cell maturation that has visible cytoplasmic evidence of hemoglobin production on a Romanowsky-stained smear?

Case Study (continued from page 59)

In addition to malaria screening, the ER physician also ordered a CBC with the following results:

WBC	14 $\times$ 10^9/L	**Differential**	
RBC	3.10 $\times$ 10^{12}/L	Segs	70%
Hb	9.2 g/dL (92 g/L)	Bands	11%
Hct	28% (0.28 L/L)	Metas	2%
MCV	93 fL	Lymphs	13%
MCH	30.6 pg/dL	Monos	2%
MCHC	32.5 g/dL	Eos	2%
PLT	230 $\times$ 10^9/L	NRBCs/100 WBCs	18
		RBC Morphology	
		Anisocytosis	3+
		Poikilocytosis	2+
		Spherocytosis	1+
		Schistocytes	1+
		Polychromasia	2+

1. Predict Stephen's reticulocyte count: low, normal, or increased.

Erythrocyte

The mature erythrocyte is a biconcave disc about 7–8 mcM in diameter and 80–100 fL in volume. It stains pink to orange because of its content of the intracellular acidophilic protein, hemoglobin (28–34 pg/cell). Mature erythrocytes lack the cellular organelles (ribosomes, mitochondria) and enzymes necessary to synthesize new lipid and protein. Thus, extensive damage to the cell membrane cannot be repaired, and the spleen will cull the damaged cell from circulation.

Erythroblastic Islands

Erythropoiesis occurs in distinctive histologic configurations called *erythroblastic islands (EIs)* that consist of concentric circles of developing erythroblasts and reticulocytes clustered around a central macrophage sometimes referred to as a "nurse cell." The central macrophage sends out slender cytoplasmic processes that maintain direct contact with each erythroblast. An array of adhesion molecules has been identified on developing erythroblasts that mediate interactions with other erythroblasts, macrophages, and bone marrow extracellular matrix, including fibronectin.[6] The maturing erythroblast moves along a cytoplasmic extension of the macrophage. As the cell becomes sufficiently mature for nuclear expulsion, cytoadhesion molecules forming macrophage/erythroblast and erythroblast/matrix attachments can become downregulated, or competing molecules can block attachment.[6] As a result, the cell detaches from the EI, passes through a pore in the cytoplasm of endothelial cells lining the marrow sinuses, and enters the circulation.[4] The central macrophage phagocytizes the nucleus, which is extruded from the orthochromatic erythroblast before the cell leaves the bone marrow. Any defective erythroblasts produced during the process of erythropoiesis ("ineffective erythropoiesis") also are phagocytized by the macrophage.

Surrounding the EI are fibroblasts, macrophages, and endothelial cells, which provide the optimal microenvironment for terminal erythroid maturation. The EI is a fragile structure and is usually disrupted by the process of marrow aspiration. However, maturing erythroblasts with adherent macrophage cytoplasmic fragments may be seen on marrow aspirate smears. Intact erythroblastic islands can occasionally be seen in marrow core biopsies.[4]

ERYTHROCYTE MEMBRANE

The red cell membrane is essential for erythrocyte development and function. Developing erythroblasts have membrane receptors for EPO and transferrin (the plasma transport protein for iron), which are required during erythropoiesis. The erythrocyte membrane selectively sequesters vital components (e.g., organic phosphates such as 2,3-BPG) and lets metabolic waste products (lactate, pyruvate) escape. The membrane helps regulate metabolism by reversibly binding and inactivating many glycolytic enzymes. It carefully balances exchange of bicarbonate and chloride ions, which aids in the transfer of carbon dioxide from tissues to lungs and balances cation and water concentrations to maintain erythrocyte ionic composition. Finally, in association with the "membrane skeleton," the erythrocyte membrane

provides the red cell with the dual characteristics of strength and flexibility needed to survive in the circulation.

Membrane Composition

The erythrocyte membrane is a phospholipid bilayer-protein complex composed of ~52% protein, 40% lipid, and 8% carbohydrate[7] (Table 5-2 ★). The chemical structure and composition control the membrane functions (e.g., transport, durability/strength, flexibility) and determine the membrane's antigenic properties. Any defect in structure or alteration in chemical composition can alter erythrocyte membrane functions and lead to the cell's premature death (Chapter 17).

Lipid Composition

Approximately 95% of the lipid content of the membrane consists of equal amounts of unesterified cholesterol and phospholipids. The remaining lipids are free fatty acids (FAs) and glycolipids (e.g., globoside). Mature erythrocytes depend on lipid exchange with the plasma and fatty acid acylation for phospholipid repair and renewal during their life span.

The overall structure of the membrane is that of a phospholipid bilayer with the phospholipid molecules arranged with polar heads exposed at the cytoplasmic and plasma membrane surfaces and their hydrophobic fatty acid side chains directed to the interior of the bilayer. The major phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidyl inositol (PI), and sphingomyelin (SM) (Table 5-2). The phospholipids are asymmetrically distributed within the membrane bilayer.[8] The choline phospholipids (PC, SM) are concentrated in the outer half of the bilayer, and the amino phospholipids (PE, PS, PI) are largely confined to the inner half. Although there is transmembrane diffusion of the phospholipids from areas of higher concentration to the bilayer leaflet of lower concentration, the asymmetry is maintained by an ATP-dependent transport system, the aminophospholipid translocase (also nicknamed "flippase").[9] Considerable evidence exists that the mobility of phospholipids within the membrane contributes to membrane fluidity. Exchange between phospholipids of the membrane and plasma can occur, especially with the phospholipids of the outer bilayer leaflet.

Cholesterol and glycolipids are intercalated between the phospholipids in the membrane bilayer.[8] Cholesterol is present in approximately equal proportions on both sides of the lipid bilayer and affects the surface area of the cell and membrane permeability. Membrane cholesterol exists in equilibrium with unesterified (free) cholesterol of plasma lipoproteins. In the plasma, cholesterol is partially converted to esterified cholesterol by the action of lecithin-cholesterol acyl transferase (LCAT). Esterified plasma cholesterol cannot exchange with the red cell membrane. When LCAT is absent (congenital LCAT deficiency or hepatocellular disease), free plasma cholesterol increases, resulting in accumulation of cholesterol within erythrocyte membranes and RBC membrane surface area expansion. An excess of cell membrane due to proportional increases in cholesterol and phospholipids, maintaining the normal ratio, results in the formation of macrocodocytes (large target cells). An increase in the cholesterol-to-phospholipid ratio, however, decreases the membrane fluidity and results in the formation of **acanthocytes** (Chapter 11). These cells have reduced survival as compared with normal RBCs.

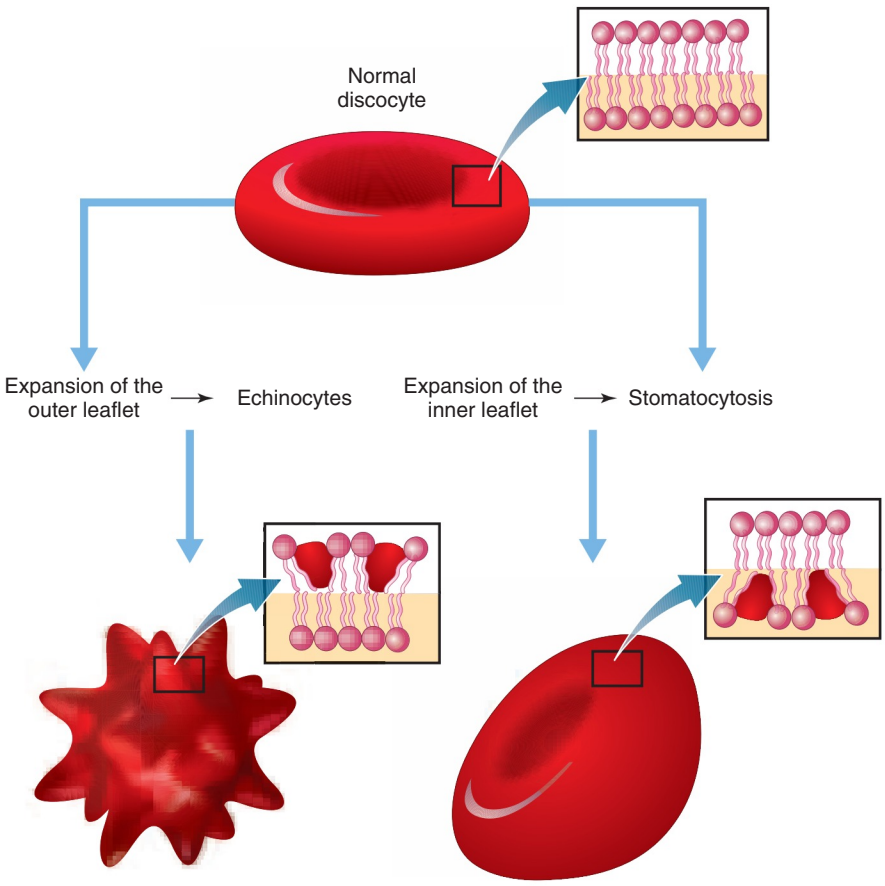
The shape of the red cell can also be altered by expansions of the separate lipid bilayers relative to each other. Processes that expand the bilayer's outer leaflet (or contract the inner) result in the formation of membrane spicules, producing echinocytes (see Figure 5-4 ■). Conversely, expansion of the inner leaflet of the bilayer leads to invagination of the membrane and the formation of stomatocytes (cup-shaped cells) (Figure 5-4).

Reticulocytes normally contain more membrane lipid and cholesterol than do mature erythrocytes. This excess lipid material is removed from the reticulocytes during the final stages of maturation in the circulation by the splenic macrophages. Splenectomized patients can have cells with an abnormal accumulation of cholesterol and/or other lipids in the membrane, which will present as target cells, acanthocytes, and/or echinocytes on the blood smear. (Alterations of red cell shape are described in Chapters 10 and 11.)

A small portion of membrane lipids consists of glycolipids in the form of glycosphingolipid. Red cell glycolipids are located entirely in the external half of the lipid bilayer with their carbohydrate portions extending into the plasma. These glycolipids are responsible for some antigenic properties of the red cell membrane (they carry the ABH, Lewis, and P blood group antigens).

★ **TABLE 5-2** Erythrocyte Membrane Composition

Lipids and glycolipids (~45%)	Unesterified cholesterol
	Phospholipids
	Phosphatidylinositol /PI
	Phosphatidylethanolamine/PE
	Phosphatidylserine/PS
	Phosphatidylcholine/PC (lecithin)
	Sphingomyelin/SM
	Lysophospholipids (lysophosphatidylcholine [LPC], lysophosphatidylethanolamine [LPE])
	Glycolipids
Proteins and glycoproteins (~55%)	Integral proteins
	Glycophorins A, B, C, D, E (carry antigens on exterior of membrane)
	Band 3 (attaches skeletal lattice to membrane lipid bilayer; anion exchange channel)
	Peripheral proteins (form membrane skeletal lattice and attach it to membrane)
	Spectrin (α and β polypeptides)
	Actin (band 5)
	Ankyrin (band 2.1)
	Band 4.2
	Band 4.1
	Adducin (band 2.9)
	Band 4.9 (dematin)
	Tropomyosin (band 7)
	Tropomodulin (band 5)



■ **FIGURE 5-4** Model of discocyte-echinocyte and discocyte-stomatocyte transformation. RBC shape is determined by the ratio of the surface areas of the two hemileaflets of the lipid bilayer. Preferential accumulation of compounds in the outer leaflet of the lipid bilayer causes expansion and results in RBC crenation and echinocytosis; expansion of the inner leaflet of the bilayer results in invagination of the membrane and stomatocytosis.

Source: Based on Clinical Expression and Laboratory Detection of Red Cell Membrane Protein Mutations by J. Palek and P. Jarolim in SEMINARS IN HEMATOLOGY 30(4):249-283, October 1993. Published by W.B./Saunders Co., an imprint of Elsevier Health Science Journals.

☑ CHECKPOINT 5-3

Explain how a deficiency or absence of LCAT can lead to the expansion of the surface area of the red cell membrane.

Protein Composition

Erythrocyte membrane proteins are of two types: integral and peripheral (Figure 5-5 ■). Integral proteins penetrate or traverse the lipid bilayer and interact with the hydrophobic lipid core of the membrane. In contrast, peripheral proteins do not penetrate into the lipid bilayer but interact with integral proteins or lipids at the membrane surface. In the red cell, the major peripheral proteins are on the cytoplasmic side of the membrane attached to membrane lipids or integral proteins by ionic and hydrogen bonds. Both types of membrane proteins are synthesized during erythroblast development. The proteins of the red cell have been studied by lysing the cell, extracting the proteins, and analyzing them by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE). The proteins, separated according to molecular weight, were identified by number according to their migration during electrophoresis with the larger proteins (which migrated the shortest distance) beginning the numbering sequence.

Integral proteins include transport proteins and the glycophorins. The three major glycophorins—A, B, and C (GPA, GPB, GPC)—are made up of three domains: the cytoplasmic, the hydrophobic, which spans the bilayer, and the extracellular on the exterior surface of the membrane.[7] The extracellular domain is heavily glycosylated and is responsible for most of the negative surface charge (zeta potential) that keeps red cells from sticking to each other and to the vessel wall. They also carry various red cell antigens (MN, Ss, U, Gerbich antigens).[8] GPC also plays a role in attaching the skeletal protein network located on the cytoplasmic side of the membrane to the lipid bilayer. The glycophorins are synthesized early in erythroid differentiation (GPC is found in BFU-E) and thus serve as lineage-specific markers for erythrocytic differentiation.

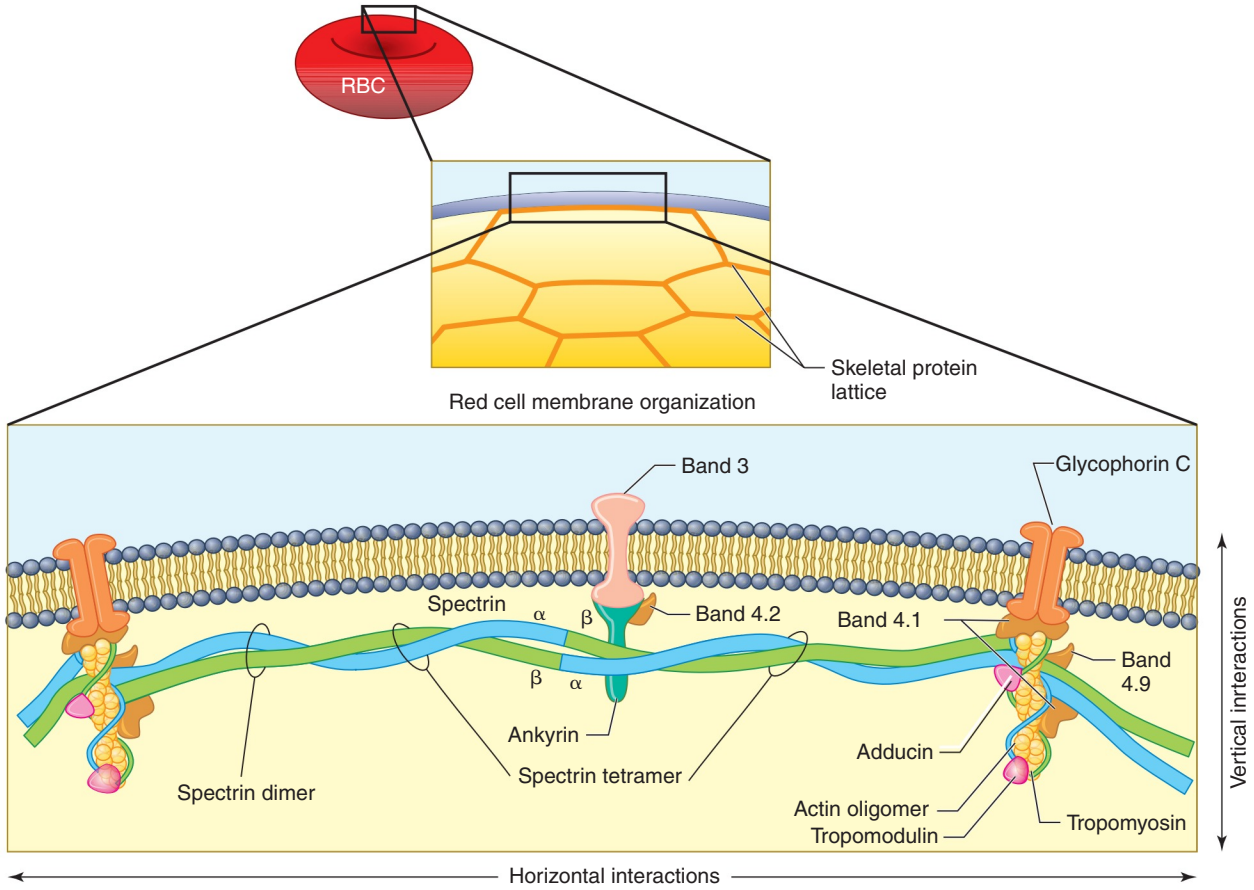


FIGURE 5-5 Model of the organization of the erythrocyte membrane showing the peripheral and integral proteins and lipids. Spectrin is the predominant protein of the skeletal protein lattice. Spectrin dimers join head to head to form spectrin tetramers. At the tail end, spectrin tetramers come together at the junctional complex. This complex is composed of actin oligomer and stabilized by tropomyosin, which sits in the groove of the actin filaments. The actin oligomer is capped on one end by tropomodulin and on the other by adducin. Band 4.9 (dematin) binds to actin and bundles actin filaments. Spectrin is attached to actin by band 4.1, which also attaches the skeletal lattice to the lipid membrane via its interaction with glycophorin C (minor attachment site). Ankyrin links the skeletal protein network to the inner side of the lipid bilayer via band 3. band 4.2 interacts with ankyrin and band 3 (major attachment site).

Band 3, also known as the *anion exchange protein 1 (AE1)*, is the major integral protein of the red cell with >1 million copies per cell. Band 3 is the transport channel for the chloride-bicarbonate exchange, which occurs during the transport of CO_2 from the tissues back to the lungs (Chapter 6). Like most transport channels, band 3 spans the membrane multiple times (12–14). Anion exchange is thought to occur by a "ping-pong" mechanism. An intracellular anion enters the transport channel and is translocated outward and released. The channel remains in the outward conformation until an extracellular anion enters and triggers the reverse cycle.[8]

In addition to its role as an anion exchanger, band 3 is a major binding site for a variety of enzymes and cytoplasmic membrane components.[8] The, cytoplasmic domain of band 3 binds glycolytic enzymes, regulating their activity; thus, band 3 serves as a regulator of red cell glycolysis. Band 3 also binds hemoglobin at its cytoplasmic domain with intact hemoglobin binding weakly but partially denatured hemoglobin (Heinz bodies) binding more avidly. Binding of hemoglobin to band 3 is thought to play a role in erythrocyte senescence. Band 3 is also important in attaching the skeletal protein network to the lipid bilayer by binding to the skeletal proteins ankyrin and band 4.2.[10] The Ii blood group antigens are carried on the carbohydrate component of the red cell band 3 protein.[11,12]

The red cell membrane contains >100 additional integral proteins.[8] These include all of the various transporter proteins (glucose transporter, urea transporter, Na^+/K^+-ATPase, Ca^{++}-ATPase, Mg^{++}-ATPase), some red cell antigens (Rh, Kell), various receptors (transferrin, insulin, etc.), and decay-accelerating factor (DAF; Chapter 17).

Peripheral proteins are found primarily on the cytoplasmic face of the erythrocyte membrane and include enzymes and structural proteins (Table 5.2). The structural proteins are organized into a two-dimensional lattice network directly laminating the inner side of the membrane lipid bilayer called the *red cell membrane skeleton*.[13] The horizontal interactions of this lattice are parallel to the membrane's plane and serve as a skeletal support for the membrane lipid layer. The vertical interactions of the lattice are perpendicular to the membrane's

plane and serve to attach the skeletal lattice network to the lipid layer of the membrane. The skeletal proteins give red cell membranes their viscoelastic properties and contribute to cell shape, deformability, and membrane stability. Defects in this cytoskeleton are associated with abnormal cell shape, decreased stability, and hemolytic anemia (Chapter 17).

Spectrin, the predominant skeletal protein, exists as a heterodimer of two large chains (α and β). The two chains associate in a side-by-side, antiparallel arrangement (N-terminal of α chain associated with C-terminal of β chain). The $\alpha\beta$ heterodimers form a slender, twisted, highly flexible molecule ~100 nm in length. Spectrin heterodimers in turn self-associate head to head to form tetramers and some larger oligomers.[8] Spectrin is described as functioning like a spring. The spectrin tetramers are tightly coiled in vivo with an end-to-end distance of only ~76 nm. (these could have an extended length of ~200 nm).[14] These coiled tetramers can extend reversibly when the membrane is stretched but cannot exceed their maximum extended length (200 nm) without rupturing.

Ankyrin is a large protein that serves as the high-affinity binding site for the attachment of spectrin to the inner membrane surface. Ankyrin binds spectrin near the region involved in dimer–tetramer associations. In turn, ankyrin is bound with high affinity to the cytoplasmic portion of band 3 (the anchor for the membrane skeleton).[15] *Band 4.2* binds to ankyrin and band 3, strengthening their interaction and helping to bind the skeleton to the lipid bilayer at its major attachment point.[10,16]

Red cell *actin* is functionally similar to actin in other cells. Red cell actin is organized into short, double-helical protofilaments of 12–14 actin monomers. These short filaments are stabilized by their interactions with other proteins of the red cell skeleton including *tropomodulin, adducin, tropomyosin*, and *band 4.9*. Spectrin dimers bind to actin filaments near the tail end of the spectrin dimer. *Band 4.1* interacts with spectrin and actin and with GPC in the overlying lipid bilayer. It serves to stabilize the otherwise weak interaction between spectrin and actin and is necessary for normal membrane stability.[8] This complex of spectrin, actin, tropomodulin, tropomyosin, adducin, band 4.9, and band 4.1 serves as the secondary attachment point for the red cell skeleton, binding to GPC of the membrane.

The skeletal proteins are in a continuous disassociation–association equilibrium with each other (e.g., spectrin dimer-tetramer interconversions) and with their attachment sites. This equilibrium occurs in response to various physical and chemical stimuli that affect the erythrocytes as they journey throughout the body. Calcium also influences the red cell cytoskeleton. Most intracellular calcium (80%) is found in association with the erythrocyte membrane. Calcium is normally maintained at a low intracellular concentration by the activity of an ATP-fueled Ca^{++} pump (Ca^{++}-ATPase). Elevated Ca^{++} levels induce membrane protein cross-linking.[17] This cross-linking essentially acts as a fixative, stabilizing red cell shape and reducing the cell's deformability. For example, the abnormal erythrocyte shape of irreversibly sickled cells (Chapter 13) can be produced by calcium-induced irreversible cross-linking and alteration of the cytoskeletal proteins.

The erythrocyte membrane together with the membrane skeleton is responsible for the dual cellular characteristics of structural integrity and deformability. The 7 mcM erythrocyte must be flexible enough to squeeze through the tiny capillary openings, particularly in the splenic vasculature (~3 mcM). At the same time, cells must be able to withstand the rigors of the turbulent circulation as they travel throughout the body. Deformability of the red cell is due to three unique cellular characteristics:[15,18]

- Its biconcave shape (large surface area-to-volume ratio)
- The viscosity of its internal contents (the "solution" of hemoglobin)
- The unique viscoelastic properties of the erythrocyte membrane

Red cells have an "elastic extension" ability, primarily due to the elasticity of coiled spectrin tetramers and association–dissociation of skeletal proteins. As a result, the cells can resume a normal shape after being distorted by an external applied force. However, application of large or prolonged forces can result in reorganization of the cytoskeletal proteins, producing a permanent deformation (e.g., dacryocytes) or, if the force is excessive, fragmentation (e.g., schistocytes).[18] In addition to being a major component of erythrocyte deformability, the membrane skeleton is the principal determinant of erythrocyte stability. The proportion of spectrin dimers versus tetramers (or higher oligomers) is a key factor influencing membrane stability.[19] Higher proportions of dimers result in increased fragility, and higher proportions of tetramers and oligomers result in stabilization. Also, interaction of the cytoskeleton with the lipid bilayer and integral membrane proteins stabilizes the cell membrane. If the bilayer uncouples from the skeleton, portions of lipid-rich membrane will be released in the form of microvesicles, resulting in a decrease in the surface area-to-volume ratio and the formation of spherocytes (Chapter 17).[20]

☑ CHECKPOINT 5-4

Compare placement in the membrane and function of peripheral and integral erythrocyte membrane proteins.

Membrane Permeability

The red cell membrane is freely permeable to water (exchanged by a water channel protein)[21] and to anions (exchanged by the anion transport protein, band 3). In contrast, the red cell membrane is nearly impermeable to monovalent and divalent cations. Glucose is taken up by a glucose transporter in a process that does not require ATP nor insulin.[22]

The cations Na^+, K^+, Ca^{++} and Mg^{++} are maintained in the erythrocyte at levels much different than those in plasma (Table 5-3 ★). Erythrocyte osmotic equilibrium is normally maintained by both the selective (low) permeability of the membrane to cations and cation

★ **TABLE 5-3** Concentration of Cations in the Erythrocyte versus Plasma

Cation	Erythrocyte (mmol/L)	Plasma (mmol/L)
Sodium (Na^+)	5.4–7.0	135–145
Potassium (K^+)	98–106	3.6–5.0
Calcium (Ca^{++})	0.0059–0.019	2.1–2.6
Magnesium (Mg^{++})	3.06	0.65–1.05

pumps located in the cell membrane. To maintain low intracellular Na^+ and Ca^{++} and high K^+ concentrations (relative to plasma concentrations), the red cell utilizes two cation pumps, both of which use intracellular ATP as an energy source.

The Na^+/K^+ cation pump hydrolyzes one mole of ATP in the expulsion of $3Na^+$ and the uptake of $2K^+$. This normally balances the passive "leaks" of each cation along its respective concentration gradient between plasma and cytoplasm. Calcium plays a role in maintaining low membrane permeability to Na^+ and K^+. An increase in intracellular Ca^{++} allows Na^+ and K^+ to move in the direction of their concentration gradients.[8] Increased intracellular Ca^{++} also activates the Gárdos channel, which causes selective loss of K^+ and, consequently, water, resulting in dehydration. Low intracellular Ca^{++} is maintained by a Ca^{++}-ATPase pump. The Ca^{++} pump depends on magnesium to maintain its transport function. Although Mg^{++} is necessary for active extrusion of Ca^{++} from the cell, Mg^{++} is not transported across the cell membrane in the process.

If erythrocyte membrane permeability to cations increases or the cation pumps fail (either due to decreased glucose for generation of ATP via glycolysis or decreased ATP), Na^+ accumulates in the cells in excess of K^+ loss. The result is an increase in intracellular monovalent cations and water, cell swelling, and, ultimately, osmotic hemolysis.

☑ CHECKPOINT 5-5

How would an increase in RBC membrane permeability affect intracellular sodium balance?

ERYTHROCYTE METABOLISM

Although the binding, transport, and release of O_2 and CO_2 are passive processes not requiring energy, various energy-dependent metabolic processes that are essential to erythrocyte viability occur. Energy is required by the erythrocyte to maintain:

1. The cation pumps, moving cations against electrochemical gradients
2. Hemoglobin iron in the reduced state
3. Reduced sulfhydryl groups in hemoglobin and other proteins
4. Red cell membrane integrity and deformability

The most important metabolic pathways in the mature erythrocyte are linked to glucose metabolism (Table 5-4 ★). Because the red cell lacks a citric acid cycle (due to the lack of mitochondria), it is limited to obtaining energy (ATP) solely by anaerobic glycolysis. Glucose enters the red cell through a membrane-associated glucose carrier in a process that does not require ATP or insulin.

Glycolytic Pathway

The erythrocyte obtains its energy in the form of ATP from glucose breakdown in the glycolytic pathway, formerly known as the Embden-Meyerhof pathway (Figure 5-6 ■). About 90–95% of the cell's glucose consumption is metabolized by this pathway. Normal erythrocytes do not store glycogen and depend entirely on plasma glucose for **glycolysis**. Glucose is metabolized by this pathway to lactate or pyruvate, producing a net gain of 2 moles of ATP per mole of glucose. If reduced nicotinamide-adenine dinucleotide (NADH) is available in the cell, pyruvate is reduced to lactate. The lactate or pyruvate formed is transported from the cell to the plasma and metabolized elsewhere in the body.

ATP is necessary to maintain erythrocyte shape, flexibility, and membrane integrity and to regulate intracellular cation concentration (see previous discussion in the section "Membrane Permeability"). Increased osmotic fragility is noted in cells with abnormal cation permeability and/or decreased ATP production. Upon the exhaustion of glucose, ATP for the cation pumps is no longer available, and cells cannot maintain normal intracellular cation concentrations. The cells become sodium and calcium loaded and potassium depleted. The cell accumulates water and changes from a biconcave disc to a sphere and is removed from the circulation.

Hexose Monophosphate (HMP) Shunt

Not all of the glucose metabolized by the red cell go through the direct glycolytic pathway. Of cellular glucose, 5 to 10% enters the oxidative HMP shunt, an ancillary system for producing reducing substances (Figure 5-6). Glucose-6-phosphate is oxidized by the enzyme

★ **TABLE 5-4** Role of Metabolic Pathways in the Erythrocyte

Metabolic Pathway	Key Enzymes	Function	Hematopathology
Glycolytic pathway	Phosphofructokinase (PFK)	Produces ATP accounting for 90% of glucose consumption in RBC	Hemolytic anemia
	Pyruvate kinase (PK)		Hereditary PK deficiency
Hexose-monophosphate shunt	Glutathione reductase (GR)	Provides NADPH and glutathione to reduce oxidants that would shift the balance of oxyhemoglobin to methemoglobin	Hemolytic anemia
	Glucose-6-phosphate dehydrogenase (G6PD)		Hereditary G6PD deficiency
			Glutathione reductase deficiency
			Hemoglobinopathies
Rapoport-Luebering	BPG-synthase	Controls the amount of 2,3-BPG produced, which in turn affects the oxygen affinity of hemoglobin	Hypoxia
Methemoglobin reductase	Methemoglobin reductase	Protects hemoglobin from oxidation via NADH (from glycolytic pathway) and methemoglobin reductase	Hemolytic anemia
			Hypoxia

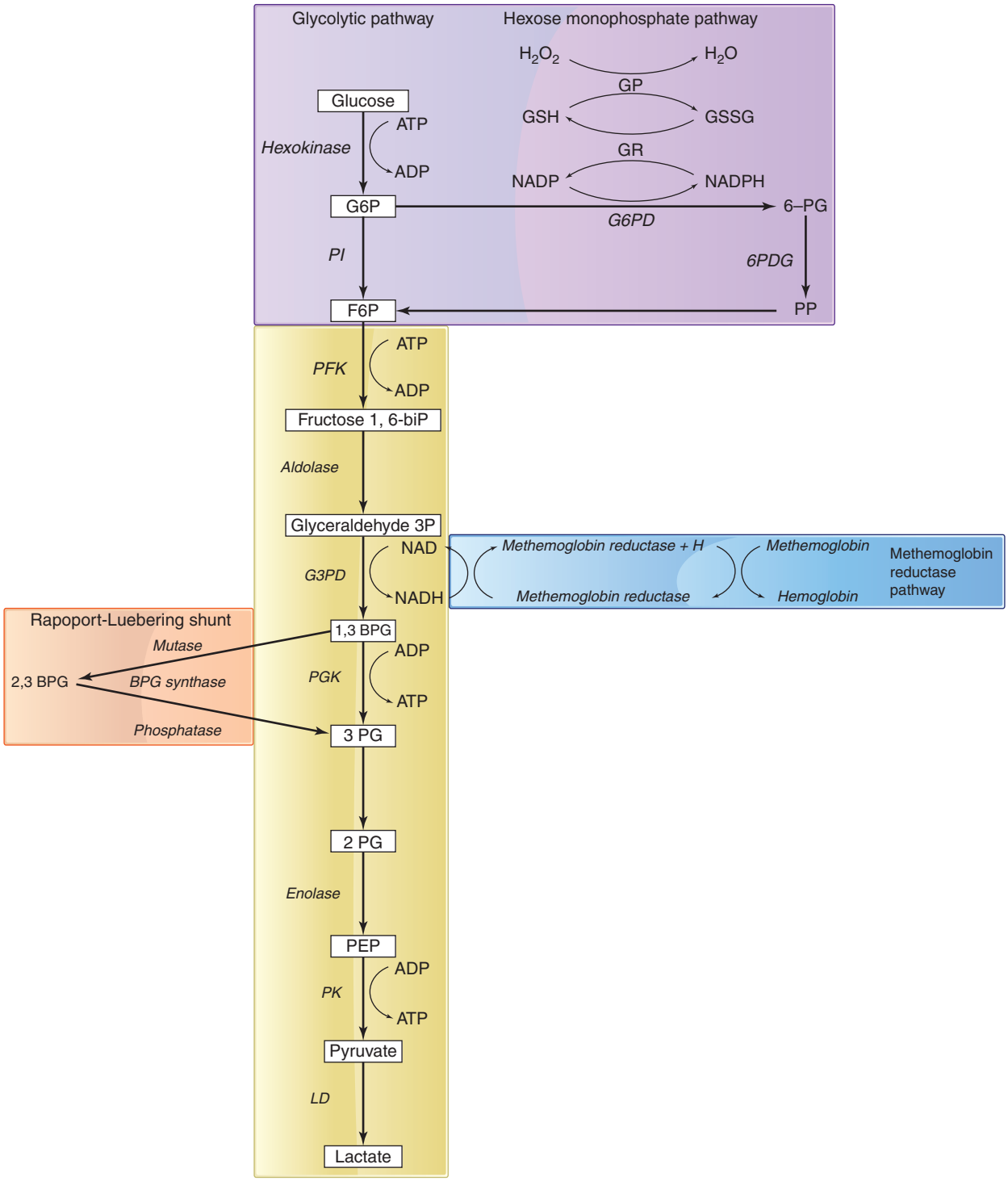


FIGURE 5-6 Erythrocyte metabolic pathways. The glycolytic pathway is the major source of energy for the erythrocyte through production of ATP. The hexose-monophosphate pathway is important for reducing oxidants by coupling oxidative metabolism with pyridine nucleotide (NADP) and glutathione (GSSG) reduction. The methemoglobin reductase pathway supports methemoglobin reduction. The Rapoport-Luebering Shunt produces 2,3-BPG, which alters hemoglobin-oxygen affinity.

G6P = glucose- 6-phosphate; PI = glucose-6-phosphate isomerase; F6P = fructose-6-phosphate; PFK = 6-phosphofructokinase; fructose 1,6-biP = fructose 1,6-bisphosphate; Glyceraldehyde 3P = glyceraldehyde 3-phosphate; G3PD = glyceraldehyde 3-phosphate dehydrogenase; 1,3 BPG = 1, 3-bisphosphoglycerate; PGK = phosphoglycerate kinase; 3PG = 3-phosphoglycerate; 2PG = 2-phosphoglycerate; PEP = phosphoenolpyruvate; PK = pyruvate kinase; LD = lactate dehydrogenase; GP = glutathione peroxidase; GR = glutathione reductase; GSH = glutathione reduced; GSSG = glutathione oxidized; G6PD = glucose-6-phosphate dehydrogenase; 6-PG = 6-phosphogluconate; 6PDG = 6-phosphodehydrogenase gluconate; PP = pentose phosphate

glucose-6-phosphate dehydrogenase (G6PD) in the first step of the HMP shunt. In the process, $NADP^+$ is reduced to nicotinamide-adenine dinucleotide phosphate NADPH.

Glutathione is highly concentrated in the erythrocyte and is important in protecting the cell from oxidant damage by reactive oxygen species (ROSs) produced during oxygen transport and by other oxidants such as chemicals and drugs. In the process of reducing oxidants, glutathione itself is oxidized. (Reduced glutathione is referred to as GSH, and the oxidized form is referred to as GSSG.) NADPH produced by the HMP shunt converts GSSG back to GSH, the form necessary to maintain hemoglobin in the reduced functional state. The erythrocyte normally maintains a large ratio of NADPH to $NADP^+$. When the HMP shunt is defective, hemoglobin sulfhydryl groups (-SH) are oxidized, which leads to denaturation and precipitation of hemoglobin in the form of a **Heinz body**. Heinz bodies attach to the inner surface of the cell membrane, decreasing cell flexibility. Macrophages in the spleen remove them from the cell together with a portion of the membrane. These bodies can be visualized with supravital stains (Chapter 37). If large portions of the membrane are damaged in this manner, the whole cell can be removed. This commonly occurs in patients with G6PD deficiency (Chapter 18).

GSH also is responsible for maintaining reduced -SH groups of cytoskeletal proteins and membrane lipids. Decreased GSH leads to oxidative injury of membrane protein -SH groups, compromising protein function and resulting in "leaky" cell membranes. Cellular depletion of ATP can then occur due to increased consumption of energy by the cation pumps.

Ascorbic acid, or vitamin C, is also an important antioxidant in the erythrocyte as it consumes oxygen free radicals and helps preserve alpha-tocopherol (vitamin E, another important antioxidant) in membrane lipoproteins.[23]

Methemoglobin Reductase Pathway

The methemoglobin reductase pathway, an offshoot of the glycolytic pathway, is essential to maintain heme iron in the reduced (ferrous) state, Fe^{++} (Figure 5-6). Methemoglobin (hemoglobin with iron in the oxidized ferric state, Fe^{+++}) is generated simultaneously with the oxidative compounds discussed earlier as O_2 dissociates from the heme iron. Methemoglobin cannot bind oxygen. The enzyme methemoglobin reductase (also known as NADH diaphorase, or cytochrome b_5) with NADH produced by the glycolytic pathway functions to reduce the ferric iron in methemoglobin, converting it back to ferrous hemoglobin. In the absence of this system, the 2% of methemoglobin formed daily eventually builds up to 20–40%, severely limiting the blood's oxygen-carrying capacity. Certain oxidant drugs can interfere with methemoglobin reductase and cause even higher levels of methemoglobin. This results in **cyanosis** (a bluish discoloration of the skin due to an increased concentration of deoxyhemoglobin in the blood).

☑ CHECKPOINT 5-6

Uncontrolled oxidation of hemoglobin results in what RBC intracellular inclusion?

Rapoport-Luebering Shunt

The Rapoport-Luebering shunt is a part of the glycolytic pathway (Figure 5-6), which bypasses the formation of 3-phosphoglycerate and ATP from 1,3-bisphosphoglycerate (1,3-BPG). Instead, 1,3-BPG forms 2,3-BPG (also known as *2,3-diphosphoglycerate, 2,3-DPG*) catalyzed by BPG mutase. Therefore, the erythrocyte sacrifices one of its two ATP-producing steps in order to form 2,3-BPG. When hemoglobin binds 2,3-BPG, oxygen release is facilitated (i.e., binding 2,3-BPG causes a decrease in hemoglobin affinity for oxygen). Thus, 2,3-BPG plays an important role in regulating oxygen delivery to the tissues (Chapter 6).

CASE STUDY (continued from page 63)

Stephen was admitted for identification and treatment of the anemia. More lab tests were ordered with the following results:

	Patient	Reference Interval
Total bilirubin	4.8 mg/dL	0.1–1.2
Direct bilirubin	1.6 mg/dL	0.1–1.0
Haptoglobin	25 mg/dL	35–165
Hemoglobin electrophoresis		
HbA	98%	>95%
Hb-F	1%	<2%
Hb-A2	1%	1.5–3.7%
Heinz body stain	Positive	Negative
Fluorescent spot test for G6PD deficiency	Positive	Negative

2. What cellular mechanism results in hemolysis due to a deficiency in G6PD?
3. Explain how Heinz body inclusions cause damage to the erythrocyte membrane.

☑ CHECKPOINT 5-7

Which erythrocyte metabolic pathway is responsible for providing the majority of cellular energy? For regulating oxygen affinity? For maintaining hemoglobin iron in the reduced state?

ERYTHROCYTE KINETICS

In the late 1800s, it was observed that individuals living at high altitudes had an increase in erythrocytes, which was attributed to an acquired adjustment to the reduced atmospheric pressure of oxygen.[24] Over the following decades, it was discovered that the stimulation of erythropoiesis in the bone marrow in response to decreased oxygen levels was the result of a hormone, erythropoietin (EPO), that is released into the peripheral blood by renal tissue in response to hypoxia. Hormonal control of red blood cell mass is closely regulated and is normally maintained in a steady state within narrow limits.

Erythrocyte Concentration

The normal erythrocyte concentration varies with sex, age, and geographic location. A high erythrocyte count 3.9–5.9 $\times$ 10^{12} L) and hemoglobin concentration (13.5–20 g/dL) at birth are followed by a gradual decrease that continues until about the second or third month of extrauterine life. At this time, red blood cell and hemoglobin values fall to 3.1–4.3 $\times$ 10^{12}/L and 9.0–13 g/dL, respectively.[25] This erythrocyte decrease in infancy is sometimes called *physiologic anemia of the newborn*, the result of a temporary cessation of bone marrow erythropoiesis after birth due to a low concentration of EPO. EPO levels are high in the fetus due to the relatively **hypoxic** environment in utero and the high oxygen affinity of hemoglobin F (fetal hemoglobin). After birth, however, when the lungs replace the placenta as the means of providing oxygen, the arterial blood oxygen saturation rises from ~45% to ~95%. EPO cannot be detected in the infant's plasma from about the first week of extrauterine life until the second or third month. Reticulocytes reflect the bone marrow activity during this time. At birth and for the next few days, the mean reticulocyte count is high (1.8–8.0%). Within a week, the count drops and remains low (<1%) until about the second month of life, at which time EPO levels rise again (when the hemoglobin levels fall to ~12 g/dL). This corresponds to the recovery from "physiologic anemia of the newborn."

Males have a higher erythrocyte concentration than females after puberty due to the presence of testosterone. Before puberty and after "male menopause," males and females have comparable erythrocyte levels.[26,27] Testosterone stimulates renal and extrarenal EPO production and directly enhances differentiation of marrow stem and progenitor cells.[28]

Individuals living at high altitudes have a higher mean erythrocyte concentration than those living at sea level. Decreases in the partial pressure of atmospheric oxygen at high altitudes result in a physiologic increase in erythrocytes in the body's attempt to provide adequate tissue oxygenation.

☑ CHECKPOINT 5-8

Why are there different reference intervals for hemoglobin concentration in male and female adults but not in male and female children?

Regulation of Erythrocyte Production

The body can regulate the number of circulating erythrocytes by changing the rate of cell production in the marrow and/or the rate of cell release from the marrow. Delivery of erythrocytes to the circulation is normally well balanced to match the rate of erythrocyte destruction, which does not vary significantly under steady-state conditions. Impaired oxygen delivery to the tissues and low intracellular oxygen tension (PO_2) trigger increased EPO release and increased erythrocyte production by the marrow. Conditions that stimulate erythropoiesis include anemia, cardiac or pulmonary disorders, abnormal hemoglobins, and high altitude. Erythropoiesis is influenced by a number of cytokines including SCF, IL-3, GM-CSF, and EPO (Chapter 4). However, EPO is the principal cytokine essential for terminal erythrocyte maturation.

EPO is a thermostable renal glycoprotein hormone with a molecular weight of about 34,000 daltons. Renal cortical interstitial cells secrete EPO in response to cellular hypoxia.[29] This feedback control of erythropoiesis is the mechanism by which the body maintains optimal erythrocyte mass for tissue oxygenation. Plasma levels of EPO are constant when the hemoglobin concentration is within the normal range but increase steeply when the hemoglobin decreases below 12 g/dL.[30] EPO is also produced by extrarenal sources, including marrow macrophages and stromal cells, which likely contribute to steady-state erythropoiesis.[2] However, under conditions of tissue hypoxia, oxygen sensors in the kidneys trigger the release of renal EPO, resulting in an increased stimulus for erythropoiesis.

EPO has been defined in biologic terms to have an activity of ~130,000 IU/mg of protein.[31] Normal plasma contains from 3 to 16 IU of EPO per L of plasma. EPO can also be found in the urine at concentrations proportional to that found in the plasma[32] (Table 5-5 ★). In anemia, EPO plasma levels are related to both hemoglobin concentration and the pathophysiology of the anemia. For example, patients with pure erythrocyte aplasia (Chapter 16) have plasma EPO levels significantly higher than patients with iron deficiency anemia or megaloblastic anemia even though hemoglobin concentration in all three types of anemia can be similar. Plasma EPO levels reflect not only EPO production but also its disappearance from the blood and/or utilization by the bone marrow (i.e., uptake by EPO-receptor-bearing cells in the marrow).

Patients with renal disease and nephrectomized patients are usually severely anemic, but they continue to make some erythrocytes and produce limited amounts of EPO in response to hypoxia. In addition to the production of EPO by marrow macrophages and stromal cells, hepatocytes act as an extrarenal source of EPO, but normally account for <15% of the total EPO production in humans.[33] The adult liver appears to require a more severe hypoxic stimulus for EPO production than the kidney. The liver is the major site of EPO production during fetal development, but at birth, a gradual shift from hepatic to renal production of EPO occurs.[34]

Increased EPO secretion is due to de novo synthesis of EPO rather than release of preformed stores. The hypoxia-induced increase

★ **TABLE 5-5** Characteristics of Erythropoietin

General Characteristics	
Composition	Glycoprotein
Stimulus for synthesis	Cellular hypoxia
Origin	Kidneys 80–90%
	Liver <15%
Reference interval	Plasma 5–30 U/L
Functions	
Stimulates BFU-E and CFU-E to divide and mature	
Increases rate of mRNA and protein (hemoglobin) synthesis	
Decreases normoblast maturation time	
Increases rate of enucleation (extrusion of nucleus)	
Stimulates early release of bone marrow reticulocytes (shift reticulocytes)	
Response to Anemia	
Generally increased except in anemia of renal disease	

of EPO is due to both increased gene transcription mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1), and stabilization of EPO messenger RNA.[35,36] Under hypoxic conditions, HIF-1 binds to DNA regulatory sequences (hypoxia-responsive element [HRE]) in the EPO gene, activating transcription. Under conditions of normal oxygen concentration, HIF-1 is degraded by a hydroxylase enzyme that requires oxygen for activity, resulting in a decreased production of EPO mRNA.[33]

EPO exerts its action by binding to specific receptors (EPO-R) on erythropoietin-responsive cells. EPO's major action is stimulation of committed progenitor cells, primarily the CFU-E, to survive, proliferate, and differentiate (see the section "Erythropoiesis and Red Blood Cell Maturation" earlier in this chapter). A small subset of BFU-E has EPO-R but in low number, and BFU-Es are largely unresponsive to the effects of EPO. Thus, under conditions of EPO stimulation, the primary elements of the erythroid precursor cells that respond are the CFU-Es and early normoblasts. However, acute demands for erythropoiesis with extremely high EPO levels can stimulate the BFU-E. When this occurs, the characteristics of the resulting erythrocytes include an increase in mean corpuscular volume (MCV) and an increase in i antigen and HbF concentration.[2] EPO-Rs on the cell membrane increase as the BFU-E matures to the CFU-E and gradually decrease as the normoblasts mature. The EPO-R is absent on reticulocytes. Other effects of EPO are described in Table 5-5.[37]

A major way by which EPO increases RBC production is by preventing apoptosis. Erythropoiesis is maintained by a finely tuned balance between the positive signals generated by EPO and negative signals from death receptor ligands and inhibitory cytokines (Chapters 2, 4). Erythroid progenitors differ in their sensitivity to EPO; some progenitors require much less EPO than others to survive and mature to reticulocytes.[38] Progenitors with increased sensitivity to EPO are thought to provide RBC production when EPO levels are normal or decreased. Progenitors that require high concentrations of EPO die of apoptosis under these conditions. Progenitors requiring high concentrations of EPO, however, will be rescued from apoptosis when EPO concentrations are elevated as occurs in anemia, thus providing increased erythrocytes under these conditions.

The EPO-R exists in the membrane as a homodimer and lacks intrinsic kinase activity. However, the cytoplasmic tail of the receptor recruits and binds cytoplasmic kinases, Janus kinases 2 (JAK-2), which are activated when EPO binds to the EPO-R (see Figure 4-7). At least four different signaling pathways are activated by this EPO/EPO-R/JAK-2 interaction. Abnormal interactions and/or function of these components have been linked to familial forms of erythrocytosis and certain myeloproliferative disorders (Chapter 24).

The normal bone marrow can increase erythropoiesis 5- to 10-fold in response to increased EPO stimulation if sufficient iron is available. Erythropoiesis is affected (and limited) by serum iron levels and by transferrin saturation[39] (Chapter 6). In hemolytic anemia, a readily available supply of iron is recycled from erythrocytes destroyed in vivo that results in a sustained an ~6-fold increase in erythropoiesis. The rate of erythropoiesis in blood loss anemia during which iron is lost from the body, however, depends more on preexisting iron stores. In this case, the rate of erythropoiesis usually does not exceed 2.5 times normal unless large parenteral or oral doses of iron are administered.

A number of tumors have been reported to cause an increase in erythropoietin production. Stimulation of the hypothalamus can cause an increase in release of EPO from the kidneys, explaining the association of polycythemia and cerebellar tumors. The serum EPO level increases dramatically in patients undergoing chemotherapy for leukemia as well as other cancers in response to marrow suppression by chemotherapeutic agents.[39]

The production of synthetic hematopoietic growth factors using recombinant DNA technology has revolutionized the management of patients with some anemias. Several recombinant forms of human EPO (rHuEPO) are available and are commonly used for treatment of the anemia associated with end-stage renal disease and chemotherapy as well as HIV-related anemia.[31,40]

CASE STUDY *(continued from page 70)*

4. Would you predict Stephen's serum erythropoietin levels to be low, normal, or increased? Why?

☑ CHECKPOINT 5-9

What would the predicted serum EPO levels be in a patient with an anemia due to end-stage kidney disease?

ERYTHROCYTE DESTRUCTION

Red blood cell destruction is normally the result of senescence. Several theories have been proposed to explain the underlying pathology of senescent red cells. Erythrocyte aging is characterized by a decline in certain cellular enzyme systems, including glycolytic enzymes and enzymes needed for maintenance of redox status. This in turn leads to decreased ATP production and loss of adequate reducing systems, resulting in oxidation of critical membrane proteins, lipids, and hemoglobin, loss of the ability to maintain cell shape and deformability, and loss of membrane integrity, all of which contribute to the cell's removal.[41,42,43] Oxidative damage also causes clustering of band 3 molecules, which can be a senescence-identifying feature. The glucose supply in the spleen is low, limiting the energy-producing process of glycolysis within the erythrocyte. Aged erythrocytes can quickly deplete their cellular level of ATP, resulting in limited ability to maintain osmotic equilibrium via the energy-dependent cation pumps. Additionally, aged erythrocytes accumulate IgG (an immunoglobulin) on their membrane. Splenic macrophages have receptors for this IgG, which can enhance recognition of aged cells. The exposure of phosphatidylserine (PS) on the outer leaflet of the erythrocyte membrane (normally concentrated on the inner leaflet of the membrane) is another signal that allows macrophages to recognize senescent erythrocytes.[44] This is the only major difference between senescent and nonsenescent erythrocytes that has been clearly documented.[45] Any combination of these events could contribute to the trapping of erythrocytes in the vasculature of the spleen and their removal by splenic macrophages.

The chromatin condensation and mitochondrial destruction that occurs during erythrocyte production parallel changes seen in apoptotic cells, as does the PS externalization seen in erythrocyte senescence. The parallels with apoptosis have led some researchers to speculate that erythrocyte maturation and senescence represent "apoptosis in slow motion."[46]

Erythrocyte removal by the spleen, bone marrow, and liver is referred to as *extravascular destruction*. This pathway accounts for about 90% of aged erythrocyte destruction. It is the most efficient method of cell removal, conserving and recycling essential erythrocyte components such as amino acids and iron. (Hemoglobin catabolism is covered in detail in Chapter 6.) Most extravascular destruction of erythrocytes takes place in the macrophages of the spleen. The spleen's architecture with its torturous circulation, sluggish blood flow, and relative hypoxic and hypoglycemic environment makes it well suited for culling aged erythrocytes and trapping those cells that have minimal defects (Chapter 3). In contrast to the macrophages in the spleen, the liver macrophages lack the ability to detect cells with minimal defects. However, because the liver receives 35% of the cardiac output (the spleen receives 5%) it can be more efficient in removing cells it recognizes as abnormal.

Severe trauma to the RBC that damages the cell's structural integrity and leads to a breach in the cell membrane results in intravascular cell lysis and release of hemoglobin directly into the blood (*intravascular destruction*). Only about 10% of erythrocyte destruction occurs in this manner. Released hemoglobin is bound by plasma proteins, and haptoglobin and hemopexin are transported to the liver where the hemoglobin is catabolized similar to the process in extravascular hemolysis.

☑ CHECKPOINT 5-10

Explain how oxidation of RBC cellular components can lead to extravascular hemolysis.

CASE STUDY (continued from page 72)

5. Stephen's haptoglobin level is 25 mg/dL. Explain why he has a low haptoglobin value.

Summary

Erythrocytes are derived from the unipotent committed progenitor cells BFU-E and CFU-E. Morphologic developmental stages of the erythroid cell include (in order of increasing maturity) the pronormoblast, basophilic normoblast, polychromatophilic normoblast, orthochromatic normoblast, reticulocyte, and erythrocyte. Erythropoietin, a hormone produced in renal tissues, stimulates erythropoiesis and is responsible for maintaining a steady-state erythrocyte mass. Erythropoietin stimulates survival and differentiation of erythroid progenitor cells, increases the rate of erythropoiesis, and induces early release of reticulocytes from the marrow.

The erythrocyte concentration varies with sex, age, and geographic location. Higher concentrations are found in males (after puberty) and newborns and at high altitudes. Decreases below the reference interval result in a condition called *anemia*.

The erythrocyte membrane is a lipid-protein bilayer complex that is important for maintaining cellular deformability and selective permeability. As the cell ages, the membrane is reduced in surface area relative to cell volume, and the cell becomes more rigid and is culled in the spleen. The normal erythrocyte life span is 100–120 days.

The erythrocyte derives its energy and reducing power from glycolysis and ancillary pathways. The glycolytic pathway provides ATP to help the cell maintain erythrocyte shape, flexibility, and membrane integrity through regulation of intracellular cation permeability. The HMP shunt provides reducing power to protect the cell from permanent oxidative injury. The methemoglobin reductase pathway helps reduce heme from the ferric (Fe^{+++}) back to the ferrous (Fe^{++}) state. The Rapoport-Luebering shunt facilitates oxygen delivery to the tissue by producing 2,3-BPG.

Destruction of aged erythrocytes occurs primarily in the macrophages of the spleen and liver through processes known as *extravascular* and *intravascular destruction*. Extravascular destruction of the erythrocyte is the major physiological pathway for aged or abnormal erythrocyte removal (splenic and hepatic macrophages).

Review Questions

Level I

1. The earliest recognizable erythroid precursor on a Wright-stained smear of the bone marrow is: (Objective 1)

   A. pronormoblast

   B. basophilic normoblast

   C. CFU-E

   D. BFU-E

2. This renal hormone stimulates erythropoiesis in the bone marrow: (Objective 4)

   A. IL-1

   B. erythropoietin

   C. granulopoietin

   D. thrombopoietin

3. An increase in 2,3-BPG occurs at high altitude in an effort to: (Objective 9)
   A. increase oxygen affinity of hemoglobin
   B. decrease oxygen affinity of hemoglobin
   C. decrease the concentration of methemoglobin
   D. protect the cell from oxidant damage
4. The erythrocyte life span is most directly determined by: (Objective 5)
   A. spleen size
   B. serum haptoglobin level
   C. membrane deformability
   D. cell size and shape
5. Which of the following depicts the normal sequence of erythroid maturation? (Objective 1)
   A. pronormoblast → basophilic normoblast → polychromatic normoblast → orthochromic normoblast → reticulocyte
   B. pronormoblast → polychromatic normoblast → orthochromic normoblast → basophilic normoblast → reticulocyte
   C. basophilic normoblast → polychromatic normoblast → reticulocyte → orthochromic normoblast → pronormoblast
   D. orthochromic normoblast → basophilic normoblast → reticulocyte → polychromatic normoblast → pronormoblast
6. The primary effector (cause) of increased erythrocyte production, or erythropoiesis, is: (Objective 4)
   A. supply of iron
   B. rate of bilirubin production
   C. tissue hypoxia
   D. rate of EPO secretion
7. An increase in the reticulocyte count should be accompanied by: (Objective 2)
   A. a shift to the left in the Hb-O_2 dissociation curve
   B. abnormal maturation of normoblasts in the bone marrow
   C. an increase in total and direct serum bilirubin
   D. polychromasia on the Wright's-stained blood smear
8. What property of the normal erythrocyte membrane allows the 7-mcM cell to squeeze through 3-mcM fenestrations in the spleen? (Objective 5)
   A. fluidity
   B. elasticity
   C. permeability
   D. deformability
9. An increase of erythrocyte membrane rigidity would be predicted to have what effect? (Objective 5)
   A. increase in erythropoietin production
   B. increase in cell volume
   C. decrease in cell life span
   D. decrease in reticulocytosis
10. Extravascular erythrocyte destruction occurs in: (Objective 7)
    A. the bloodstream
    B. macrophages in the spleen
    C. the lymph nodes
    D. bone marrow sinuses

Level II

1. Results of a CBC revealed a MCHC of 40 g/dL. What characteristic of the RBC will this affect? (Objective 2)
   A. oxygen affinity
   B. cell metabolism
   C. membrane permeability
   D. cell deformability
2. If the erythrocyte cation pump fails because of inadequate generation of ATP, the result is: (Objective 3)
   A. decreased osmotic fragility due to formation of target cell
   B. formation of echinocytes due to influx of potassium
   C. cell crenation due to efflux of water and sodium
   D. cell swelling due to influx of water and cations
3. As a person ascends to high altitudes, the increased activity of the Rapoport-Luebering pathway: (Objective 4)
   A. causes precipitation of hemoglobin as Heinz bodies
   B. has no effect on oxygen delivery to tissues
   C. causes increased release of oxygen to tissues
   D. causes decreased release of oxygen to tissues
4. A newborn has a hemoglobin level of 16.0 g/dL at birth. Two months later, a CBC indicates a hemoglobin concentration of 11.0 g/dL. The difference in hemoglobin concentration is most likely due to: (Objective 1)
   A. chronic blood loss
   B. inherited anemia
   C. increased intravascular hemolysis
   D. physiologic anemia of the newborn

5. A 50-year-old patient had a splenectomy after a car accident that damaged her spleen. She had a CBC performed at her 6-week postsurgical checkup. Many target cells were identified on the blood smear. This finding is most likely: (Objective 2)

   A. an indication of liver disease

   B. a loss of RBC membrane peripheral proteins

   C. an abnormal protein to phospholipid ratio of the RBC membrane

   D. an accumulation of cholesterol and phospholipid in the RBC membrane

6. Which of the following is necessary to maintain reduced levels of methemoglobin in the erythrocyte? (Objective 4)

   A. vitamin B_6

   B. NADH

   C. 2,3-BPG

   D. lactate

7. A patient lost about 1500 mL of blood during surgery but was not given blood transfusions. His hemoglobin before surgery was in the reference range. The most likely finding 3 days later would be: (Objective 1, 6)

   A. increase in total bilirubin

   B. increase in indirect bilirubin

   C. increase in erythropoietin

   D. increased haptoglobin

8. A patient with kidney disease has a hemoglobin of 8 g/dL. This is most likely associated with: (Objective 6)

   A. decreased EPO production

   B. increased intravascular hemolysis

   C. abnormal RBC membrane permeability

   D. RBC fragility due to accumulation of intracellular calcium

9. A laboratory professional finds evidence of Heinz bodies in the erythrocytes of a 30-year-old male. This is evidence of: (Objective 4)

   A. increased oxidant concentration in the cell

   B. decreased hemoglobin-oxygen affinity

   C. decreased production of ATP

   D. decreased stability of the cell membrane

10. A 65-year-old female presents with an anemia of 3 weeks' duration. In addition to a decrease in her hemoglobin and hematocrit, she has a reticulocyte count of 6% and 3+ polychromasia on her blood smear. Based on these preliminary findings, what serum erythropoietin result is expected? (Objective 6)

    A. decreased

    B. normal

    C. increased

    D. no correlation

Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

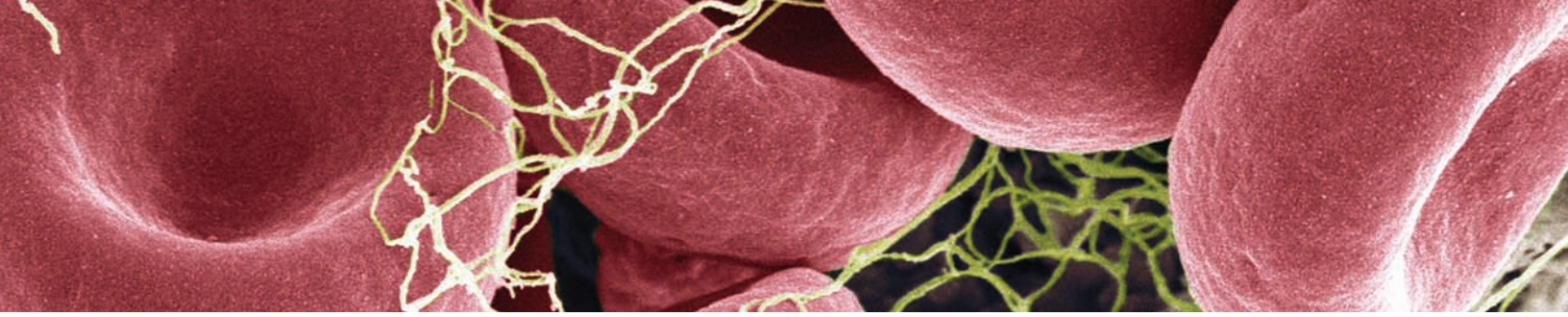
Disclaimer

The views expressed in this chapter are those of the author and do not necessarily reflect the official policy or position of the Department of the Army, the Department of Defense, or the U.S. government.

References

1. Beutler E. The red cell: a tiny dynamo. In: Wintrobe, MM, ed. *Blood, Pure and Eloquent*. New York: McGraw-Hill; 1980:141–68.
2. Papayannopoulou T, Migliaccio AR, Abkowitz JL et al. Biology of erythropoiesis, erythroid differentiation and maturation. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:276–94.
3. Gregg XT, Prchal JT. Anemia of endocrine disorders. In: Kaushansky K, Lichtman MA, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010;509–11.
4. Bull BS, Herrmann PC. Morphology of the erythron. In: Kaushansky K, Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Prchal JT, eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010;409–27.
5. Jing L, Guo X, Mohandas N, Chasis JA, An X. Membrane remodeling during reticulocyte maturation. *Blood*. 2010;115(10);2021–27.
6. Chasis JA, Mohandas N. Erythroblastic Islands: niches for erythropoiesis. *Blood*. 2008;112(3);470–78.
7. Mohandas N. The red cell membrane. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*. Philadelphia: Elsevier Churchill Livingstone; 1995:264–69.

8. Lux SE, Palek J. Disorders of the red cell membrane. In: Handin RI, Lux SE, Stossel TP, eds. *Blood Principles and Practice of Hematology*. Philadelphia: J. B. Lippincott; 1995:1701–1818.
9. Devaux PF. Protein involvement in transmembrane lipid asymmetry. *Annu Rev Biophys Biomol Struct*. 1992;21:417–39.
10. Butler J, Mohandas N, Waugh RE. Integral protein linkage and the bilayer-skeletal separation energy in red blood cells. *Biophys J*. 2008;95(4);1826–36.
11. Fukuda M, Dell A, Fukuda MN. Structure of fetal lactosaminoglycan: The carbohydrate moiety of band 3 isolated from human umbilical cord erythrocytes. *J Biol Chem*. 1984;259:4782–91.
12. Fukuda M, Dell A, Oates JE et al. Structure of branched lactosaminoglycan, the carbohydrate moiety of band 3 isolated from adult erythrocytes. *J Biol Chem*. 1984;259:8260–73.
13. Platt OS. Inherited disorders of red cell membrane proteins. In: Nagel RL, ed. *Genetically Abnormal Red Cells*. Boca Raton, FL: CRC Press; 1988.
14. Vertessy BG, Steck TL. Elasticity of the human red cell membrane skeleton: Effects of temperature and denaturants. *Biophys J*. 1989;55:255–62.
15. Bennett V, Stenbuck PJ. The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. *Nature*. 1979;280:468–73.
16. Cohen CM, Dotimas E, Korsgren C. Human erythrocyte membrane protein band 4.2 (pallidin). *Semin Hematol*. 1993;30:119–37.
17. Lorand L, Siefring GE Jr, Lowe-Krentz L. Enzymatic basis of membrane stiffening in human erythrocytes. *Semin Hematol*. 1979;16:65–74.
18. Mohandas N, Chasis JA. Red cell deformability, membrane material properties, and shape: regulation by transmembrane, skeletal, and cytosolic proteins and lipids. *Semin Hematol*. 1993;30:171–92.
19. Liu SC, Pallek J. Spectrin tetramer-dimer equilibrium and the stability of erythrocyte membrane skeletons. *Nature*. 1980;285:586–88.
20. Liu S-C, Derick LH. Molecular anatomy of the red blood cell membrane skeleton: structure-function relationships. *Semin Hematol*. 1992;29: 231–43.
21. Zeidel ML, Ambudkar SV, Smith BL et al. Reconstitution of functional water channels in liposomes containing purified red cell CHIP28 protein. *Biochemistry*. 1992;31:7436–40.
22. Mueckler M, Caruso C, Baldwin SA et al. Sequence and structure of a human glucose transporter. *Science*. 1983;229:941–45.
23. May JM. Ascorbate function and metabolism in the human erythrocyte. *Frontiers in Bio*. 1998;3(1):1–10.
24. Viault F. Sur l'augmentation considerable du nombre des globules ranges dans le sang chez les habitants des haute plateaux de l'amerique du sud. *CR Acad Sci (Paris)*. 1890;119:917–18.
25. Bao W, Dalferes ER Jr, Srinivasan SR. Normative distribution of complete blood count from early childhood through adolescence: the Bogalusa Heart Study. *Prev Med*. 1993;22:825–37.
26. Cavalieri TA, Chopra A, Bryman PN. When outside the norm is normal: interpreting lab data in the aged. *Geriat*. 1992;47:66–70.
27. Kosower NS. Altered properties of erythrocytes in the aged. *Am J Hematol*. 1993;42:241–47.
28. Besa EC. Hematologic effects of androgens revisited: an alternative therapy in various hematologic conditions. *Semin Hematol*. 1994;31:134–45.
29. Bachmann S, Le Hir M, Eckardt KU. Co-localization of erythropoietin mRNA and ecto-5′-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. *J Histochem Cytochem*. 1993;41:335–41.
30. Gabrilove J. Overview: Erythropoiesis, anemia, and the impact of erythropoietin. *Sem Hematol*. 2000;l37(4)(supp):1–3.
31. Jelkmann W. Erythropoietin after a century of research: younger than ever. *Eur J Haematol*. 2007;78:183–205.
32. Marsden JT. Erythropoietin measurement and clinical application. *Ann Clin Biochem*. 2006;43:97–104.
33. Hodges VM, Rainey S, Lappin TR et al. Pathophysiology of anemia and erythrocytosis. *Crit Rev Oncol Hematol*. 2007;64:139–58.
34. Zanjani ED, Ascensao JL, McGlave PB et al. Studies on the liver to kidney switch of erythropoietin production. *J Clin Invest*. 1981;67:1183–88.
35. Nangaku M, Eckardt KU. Hypoxia and the HIF system in kidney disease. *J Mol Med*. 2007;85:1325–30.
36. Wang GL, Semenza GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol*. 1992;12:5447–54.
37. Unger EF, Thompson AM, Blank MJ et al. Erythropoiesis-stimulating agents: time for a reevaluation. *N Engl J Med*. 2010;362;189–92.
38. Kelley LL, Koury MJ, Bondurant MC et al. Survival or death of individual proerythroblasts results from differing erythropoietin sensitivities: a mechanism for controlled rates of erythrocyte production. *Blood*. 1993;82:2340–52.
39. Swabe Y, Takiguchi Y, Kikuno K et al. Changes in levels of serum erythropoietin, serum iron, and unsaturated iron binding capacity during chemotherapy for lung cancer. *J Clin Onc*. 1998;28(3):182–86.
40. Kimmel PL, Greer JW, Milam RA et al. Trends in erythropoietin therapy in the U.S. dialysis population. *Semin Nephrol*. 2000;20(4):335–44.
41. Suzuki T, Dale GI. Senescent erythrocytes: isolation of in vivo aged cells and their biochemical characteristics. *Proc Natl Acad Sci USA*. 1998;85:1647–51.
42. Zimran A, Forman L, Suzuki T et al. In vivo aging of red cell enzymes: study of biotinylated red cells in rabbits. *Am J Haematol*. 1990;33:249–54.
43. Steinberg MH, Benz EJ Jr, Adewoye AH et al. Pathobiology of the human erythrocyte and its hemoglobins. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:427–38.
44. Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci USA*. 1998;95:3077–81.
45. Beutler E. Destruction of erythrocytes. In: Kaushansky K, Lichtman MA, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010;449–54.
46. Gottlieb RA. Apoptosis. In: Kaushansky K, Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Prchal JT, eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010;161–67. http://accessmedicine.mhmedical.com.libproxy.uthscsa.edu/content.aspx?bookid=358&sectionid=39835828.



6 Hemoglobin

SHIRLYN B. MCKENZIE, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Diagram the quaternary structure of a hemoglobin molecule, identifying the heme ring, globin chains, and iron.
2. Assemble fetal and adult hemoglobin molecules with appropriate globin chains.
3. Explain how pH, temperature, 2,3-BPG, and PO_2 affect the oxygen dissociation curve (ODC).
4. List the types of hemoglobin normally found in adults and newborns and give their approximate concentration.
5. Summarize hemoglobin's function in gaseous transport.
6. Define hemoglobin reference intervals
7. Explain how the fine balance of hemoglobin concentration is maintained.
8. Compare HbA with HbA1c and explain what an increased concentration of HbA1c means.
9. Diagram and describe the mechanism of extravascular erythrocyte destruction and hemoglobin catabolism and name laboratory tests that can be used to evaluate it.
10. Diagram and describe the mechanism of intravascular erythrocyte destruction and hemoglobin catabolism and name laboratory tests that can be used to evaluate it.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Construct a diagram to show the synthesis of a hemoglobin molecule.
2. Describe the ontogeny of hemoglobin types; contrast differences in oxygen affinity of HbF and HbA and relate them to the structure of the molecule.
3. Explain the molecular control of heme synthesis.
4. Given information on pH, 2,3-BPG, CO_2, temperature, and HbF concentration; interpret the ODC, and translate it into the physiologic effect on oxygen delivery.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

5. Contrast the structures and functions of relaxed and tense hemoglobin and propose how these structures affect gaseous transport.
6. Describe how abnormal hemoglobins are acquired, and select a method by which they can be detected in the laboratory.
7. Assess the oxygen affinity of abnormal, acquired hemoglobins and reason how this affects oxygen transport.
8. Compare and contrast the exchange of O_2, CO_2, H^+, and Cl^- at the level of capillaries and the lungs.
9. Explain the role of hemoglobin in the NO-control of blood flow and vessel homeostasis.
10. Compare and contrast erythrocyte extravascular destruction with intravascular destruction and identify which process is dominant given laboratory results.
11. Identify the breakdown products of hemoglobin and determine how the body conserves and recycles essential components.

Key Terms

Artificial oxygen carrier (AOC)
Bilirubin
Bohr effect
Carboxyhemoglobin
Chloride shift
Cyanosis
Deoxyhemoglobin
Ferritin
Glycosylated hemoglobin
Haptoglobin
Heme
Hemopexin
Hemosiderin
Hemosiderinuria
Hypoxia
Oxygen affinity
Oxyhemoglobin
Methemoglobin
Relaxed (R) structure
Sulfhemoglobin
Tense (T) structure

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- List and describe the stages of erythrocyte maturation (Chapter 5).
- Summarize the role of erythropoietin in erythropoiesis (Chapter 5).
- Describe the site of erythropoiesis (Chapter 4).

Level II

- Describe the metabolic pathways present in the mature erythrocyte, and explain their role in maintaining viability of the erythrocyte (Chapter 5).
- Summarize the development of hematopoiesis from the embryonic stage to the adult (Chapter 4).

CASE STUDY

We will address this case study throughout the chapter.

Jerry, a 44-year-old male, arrived in the emergency room by ambulance after a bicycle accident. Examination revealed multiple fractures of the femur. He was otherwise healthy. The next day, he was taken to surgery to repair the fractures. After surgery, his hemoglobin was 7 g/dL. He refused blood transfusions and was discharged 6 days later. Jerry called his doctor within days of being discharged and told him that he had difficulty walking around the house on crutches because of shortness of breath and lack of stamina.

Consider why Jerry's hemoglobin decreased after surgery and how this could be related to his current symptoms.

OVERVIEW

This chapter describes the synthesis and structure of hemoglobin and factors that regulate its production. It compares the different types of hemoglobin produced according to developmental stage, considers the function of hemoglobin in gaseous exchange, and analyzes factors that affect this function. The chapter also discusses structure, formation, and laboratory detection of abnormal hemoglobins.

INTRODUCTION

Hemoglobin is a highly specialized intracellular erythrocyte protein responsible for transporting oxygen from the lungs to tissue for oxidative metabolism and facilitating carbon dioxide transport from the tissue to the lungs. Each gram of hemoglobin can carry 1.34 mL of oxygen. It is also a transporter of nitric oxide, which modulates vascular tone.

Hemoglobin occupies approximately 33% of the volume of the erythrocyte and accounts for 90% of the cell's dry weight. Each cell contains between 28 and 34 pg of hemoglobin, a concentration close to the solubility limit of hemoglobin. This concentration is measured by cell analyzers and reported as mean corpuscular hemoglobin (MCH). In anemic states, the erythrocyte can contain less hemoglobin (decreased MCH) and/or the individual can have fewer erythrocytes present, both of which result in a decrease of the blood's oxygen-carrying capacity.

The erythrocyte's membrane and its metabolic pathways are responsible for protecting and maintaining the hemoglobin molecule in its functional state. Abnormalities in the membrane that alter its permeability or alterations of the cell's enzyme systems can lead to

changes in the structure and/or function of the hemoglobin molecule and affect the capacity of this protein to deliver oxygen.

Although a small amount of hemoglobin is synthesized as early as the pronormoblast stage, most hemoglobin synthesized in the developing normoblasts occurs at the polychromatophilic normoblast stage. In total, 75–80% of the cell's hemoglobin is made before the extrusion of the nucleus. Because the reticulocyte does not have a nucleus, it cannot make new RNA for protein synthesis. However, residual RNA and mitochondria in the reticulocyte enable the cell to make the remaining 20–25% of the cell's hemoglobin. The mature erythrocyte contains no nucleus, ribosomes, or mitochondria and is unable to synthesize new protein.

Hemoglobin concentration in the body is the result of a fine balance between production and destruction of erythrocytes. The normal hemoglobin concentration in an adult male is about 15 g/dL with a total blood volume of about 5 L (50 dL). Therefore, the total body mass of hemoglobin is approximately 750 g:

$$15 \text{ g/dL} \times 50 \text{ dL} = 750 \text{ g}$$

Because the normal erythrocyte life span is ~120 days, $^{1}/_{120}$ of the total amount of hemoglobin is lost each day through removal of senescent erythrocytes. Thus, an equivalent amount must be synthesized each day to maintain a steady-state concentration. This amounts to approximately 6 g of new hemoglobin per day:

$$\frac{750 \text{ g}}{120 \text{ days}} = 6.25 \text{ g/day} \quad \begin{matrix}\text{(amount of hemoglobin lost} \\ \text{and synthesized each day)}\end{matrix}$$

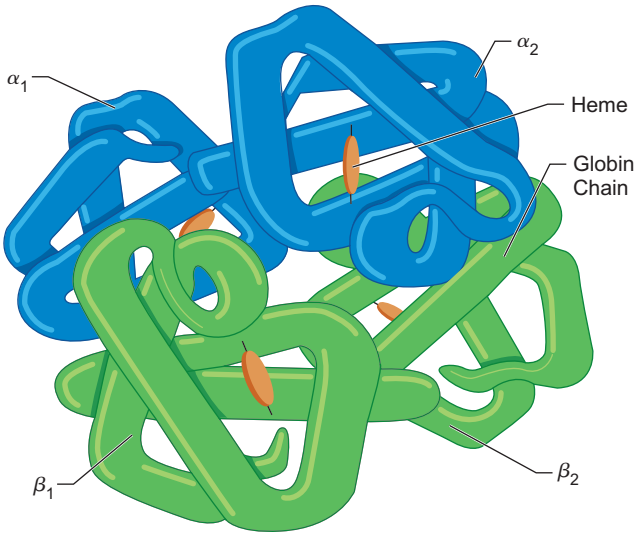
If we divide the total amount of hemoglobin synthesized each day (6.25 g) by the mean amount of hemoglobin in a red cell (MCH, ~30 pg), we can determine the daily production of new red blood cells:

$$\frac{6.25 \text{ g/day}}{30 \text{ pg/cell}} \times \frac{10^{12} \text{ pg}}{\text{g}} = 2 \times 10^{11} \text{ cells/day}$$

HEMOGLOBIN STRUCTURE

Hemoglobin is a large tetrameric molecule, molecular weight 66,700 daltons, composed of four globular protein subunits (Figure 6-1 ■). Each of the four subunits contains a heme group and a globin chain. Heme, the prosthetic group of hemoglobin, is a tetrapyrrole ring with ferrous iron located in the center of the ring. Hemoglobin structure is described in Table 6-1 ★ (Figure 6-2 ■). Each heme subunit can carry one molecule of oxygen bound to the central ferrous iron; thus, each hemoglobin molecule can carry four molecules of oxygen.

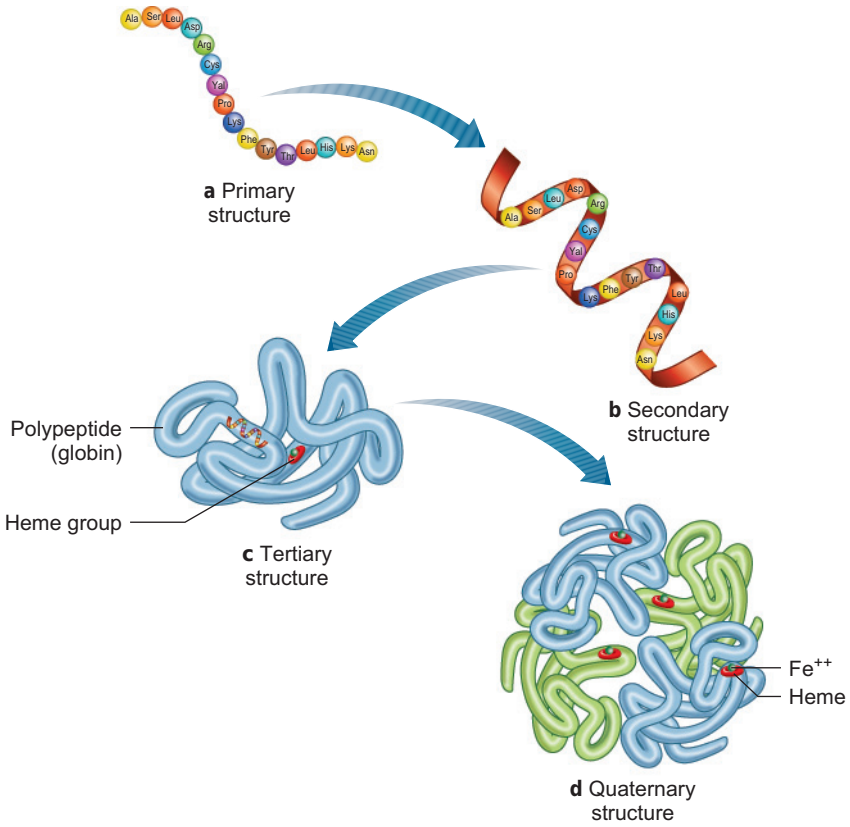
The composition of the globin chains is responsible for the different functional and physical properties of hemoglobin. Two types of globin chains are produced: alpha-like (alpha [α], zeta [ζ]), and non-alpha (epsilon [ε], beta [β], delta [δ], gamma [γ]). The tetrameric hemoglobin molecule consists of two pairs of unlike chains: two identical α-like and two identical non-α-chains. A pair of α-like chains (α or ζ) combines with a pair of non-α-chains (ε, β, δ, or γ) to form the various types of hemoglobin (Table 6-2 ★). The arrangement of each globin chain is similar. Each α- and ζ-chain has 141 amino acids, and each ε-, β-, δ-, and γ-chain has 146 amino acids. The β- chain is composed of eight α-helical segments separated by seven short, nonhelical segments, and the α-chain has seven α-helical segments. The helical segments are lettered A–H, starting at the amino



■ **FIGURE 6-1** Hemoglobin is a molecule composed of four polypeptide subunits. Each subunit has a globin chain with a heme nestled in a hydrophobic crevice that protects the iron from being oxidized. There are four different types of globin chains—α, β, δ, γ. Two α-chains and two non-α-chains occur in identical pairs to form a tetramer. The types of globin chains present determine the type of hemoglobin. Depicted here is hemoglobin A, consisting of two α- and two β-chains. The contacts between the α, β-chains in a dimer (i.e., $\alpha_1\beta_1$) are extensive and allow little movement. The contacts between the dimer pairs (i.e., $\alpha_1\beta_2$, $\alpha_2\beta_1$), however, are smaller and allow conformational change of the molecule as it goes from oxyhemoglobin to deoxyhemoglobin.

★ **TABLE 6-1** The Structure of Hemoglobin

Structure	Conformational Description
Primary	Sequence of individual amino acids held together by peptide bonds in the globin chains; is critical to stability and function of molecule; determines the overall structure
Secondary	Arrangement of the amino acids resulting from hydrogen bonding between the peptide bonds of the amino acids next to or near each other (75% of the chain is in the form of an α-helix; each chain consists of 7 or 8 α-helix segments, labeled A → H, separated by nonhelical [pleated] segments that do not participate in forming the α-helix but allow the polypeptide to fold on itself)
Tertiary	Folding superimposed on the helical and pleated domains; forms the heme hydrophobic pocket within globin chains and places polar (hydrophilic) residues on the exterior of the molecule; this tertiary structure changes upon ligand binding
Quaternary	Relationship of the four protein subunits to one another; quaternary structural changes that occur upon ligand binding result from the tertiary changes



■ **FIGURE 6-2** The structure of hemoglobin. **(a)** Primary structure is the sequence of amino acids. **(b)** Secondary structure is the coiled α-helix and β-pleated sheet formed by hydrogen bonding between the peptide bonds in the chain. **(c)** Tertiary structure is the folding of the molecule into a three-dimensional structure. **(d)** Quaternary structure is the combination of the four polypeptide subunits, each of which contains a heme group, into a larger protein.

end of the chain. The amino acids of the globin chains are identified by their helix location and amino acid number (e.g., F8 is the eighth amino acid in the F helix). Amino acids between helices are identified by amino acid number and the letters of the two helices (e.g., EF3). The nonhelical segments allow the chains to fold upon themselves.

The four subunits of hemoglobin, each consisting of a heme group surrounded by a globin chain, are held together by salt bonds, hydrophobic contacts, and hydrogen bonds in a tetrahedral formation giving the hemoglobin molecule a nearly spherical shape. When ligands such as oxygen bind to hemoglobin, the number and stringency of intersubunit contacts change and the shape of the molecule changes. Mutations in the primary structure of globin chains can affect subunit or dimer pair interactions and thus alter hemoglobin-oxygen affinity or the molecule's stability.

☑ CHECKPOINT 6-1

Describe the quaternary structure of a molecule of hemoglobin. How can a mutation in one of the globin chains at the subunit interaction site, $\alpha_1\beta_2$, affect hemoglobin function?

★ **TABLE 6-2** Normal Types of Hemoglobin According to Developmental Stage

Developmental Stage	Type	Globin Chains	Reference Interval
Embryonic	Gower 1	$\zeta_2\varepsilon_2$	—
	Gower 2	$\alpha_2\varepsilon_2$	—
	Portland	$\zeta_2\gamma_2$	—
Fetal	HbF	$\alpha_2\gamma_2$	90–95% before birth
			50–85% at birth
	HbA	$\alpha_2\beta_2$	10–40% at birth
	HbA_2	$\alpha_2\delta_2$	<1% at birth
>1 year old	HbF	$\alpha_2\gamma_2$	<2%
	HbA	$\alpha_2\beta_2$	>95%
	HbA_2	$\alpha_2\delta_2$	<3.5%
Adult	HbA	$\alpha_2\beta_2$	>95%
	HbA_2	$\alpha_2\delta_2$	1.5–3.7%
	HbF	$\alpha_2\gamma_2$	<2%

α = alpha; β = beta; γ = gamma; δ = delta; ζ = zeta

HEMOGLOBIN SYNTHESIS

Heme

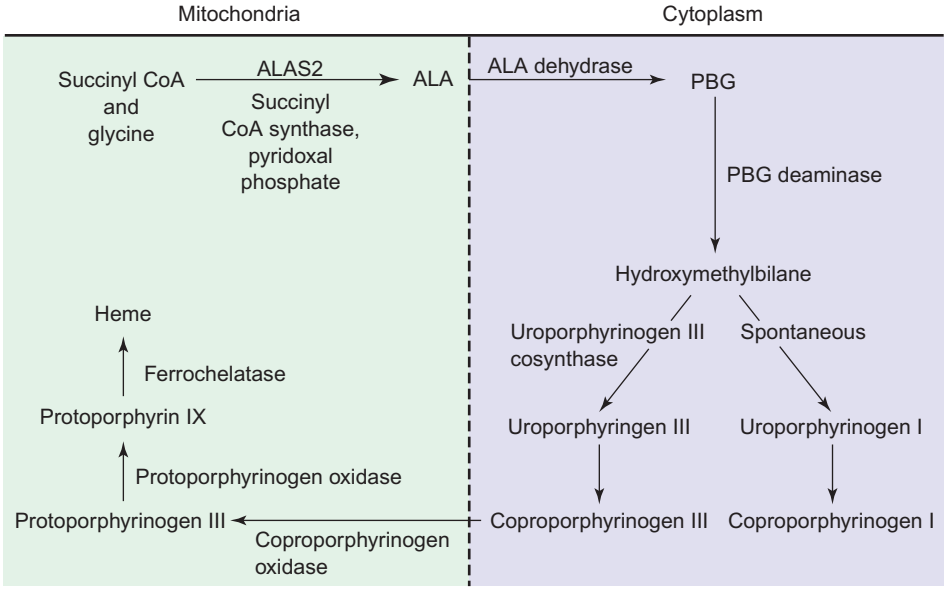
Heme is an iron-chelated porphyrin ring that functions as a prosthetic group (nonamino acid component) of a protein. The porphyrin ring, protoporphyrin IX, is composed of a flat tetrapyrrole ring with ferrous iron (Fe^{++}) inserted into the center. (Porphyrins are metabolically active only when chelated.) Ferrous ions have six electron pairs per atom. In heme, four of these electron pairs are coordinately bound to the N atoms of each of the four pyrrole rings. In hemoglobin, one of the two remaining electron pairs (fifth) is coordinately bound with the N of the proximal histidine (F8) of the globin chain, and the other pair (sixth) is the binding site for molecular oxygen. In the deoxygenated state, the sixth electron pair is occupied by a water molecule. Iron must be in the ferrous (Fe^{++}) state for oxygen binding to occur. Ferric iron (Fe^{+++}), which has lost an electron, cannot serve as an oxygen carrier because the sixth potential binding site (electron pair) for oxygen is no longer available.

Heme synthesis begins in the mitochondria with the condensation of glycine and succinyl coenzyme A (CoA) to form 5-aminolevulinic acid (ALA). This reaction occurs in the presence of the cofactor pyridoxal phosphate and the enzyme 5-aminolevulinate synthase (ALAS). This first reaction is the rate-limiting step in the synthesis of heme and occurs only when the cell has an adequate supply of iron[1] (Chapter 12). Synthesis continues through a series of steps in the cytoplasm, eventually forming coproporphyrinogen. Coproporphyrinogen then reenters the mitochondria and is further modified to form the protoporphyrin IX ring (Figure 6-3 ■). The final step, also occurring in the mitochondria, is the chelation of iron with protoporphyrin IX catalyzed by ferrochelatase to form heme (Figure 6-4 ■). Heme then leaves the mitochondria to combine with a globin chain in the cytoplasm. See Web Figure 6-1 for detailed molecular structures of intermediates in heme synthesis.

Globin Chain Synthesis

Globin chain synthesis is directed by genes in two clusters on chromosomes 11 and 16 (Figure 6-5 ■). These genes produce the seven different types of globin chains: zeta, alpha, epsilon, gamma-A, gamma-G, delta, and beta ($\alpha, \zeta, \epsilon, \gamma^A, \gamma^G, \delta, \beta$). Two are found only in embryonic hemoglobins (ζ, ε). The genes for the ζ-chain (the fetal equivalent of the α-chain) and α-chain are located on the short arm of chromosome 16 (the α gene cluster). The ζ-chain is synthesized very early in embryonic development, but after 8–12 weeks, ζ-chain synthesis is replaced by α-chain synthesis. There are two α-loci (α-1, α-2), both of which transcribe mRNA for α-chain synthesis. The protein product from each locus is structurally identical. The non-α-globin genes are arranged in linear fashion in order of activation on chromosome 11 (the non-α-gene cluster).

The ε-gene, the first non-α-gene to be activated, is located toward the 5′ end of chromosome 11; during embryonic development, ε-chain synthesis is switched off, and the two γ-genes are activated. One γ-gene directs the production of a γ-chain with glycine at amino acid position 136, γ^G, and the other directs the production of a γ-chain with alanine at position 136, γ^A. The γ^G-chain synthesis predominates before birth (3:1), but γ^G- and γ^A-chain syntheses are equal (1:1) in adults. The next two genes on chromosome 11, δ and β,



■ **FIGURE 6-3** Synthesis of heme. It begins in the mitochondria with the condensation of glycine and succinyl CoA catalyzed by 5-aminolevulinate synthase 2 (ALAS2) and succinyl CoA synthase and co-factor pyridoxal phosphate. The product, 5-aminolevulinate (ALA), leaves the mitochondria to form the pyrrole ring, porphobilinogen (PBG). The combination of four pyrroles to form a linear tetrapyrrole (hydroxymethylbilane), the cyclizing of the linear form to uroporphyrinogen, and the decarboxylation of the side chains to form coproporphyrinogen occur in the cytoplasm. The final reactions, the formation of protoporphyrin IX, and the insertion of iron into the protoporphyrin ring occur in the mitochondria.

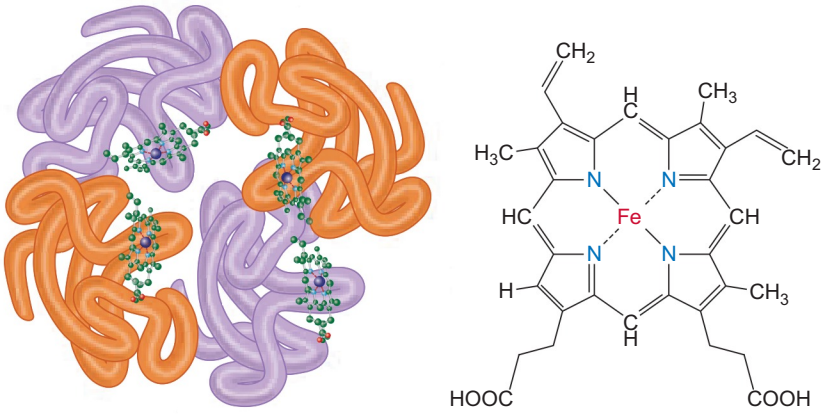


FIGURE 6-4 The hemoglobin molecule on the left reveals the quartenary structure of hemoglobin with four protein chains, each folded around a heme molecule. On the right is a heme molecule. Heme is composed of a flat tetrapyrrole ligand (porphyrin) and iron. The iron has six coordinate sites. Nitrogen atoms of porphyrin occupy four coordination sites in a square planar arrangement around the iron. Iron in the ferrous state has two other coordinate sites, one of which is occupied by the N of the proximal histidine (F8) of globin and one with molecular oxygen or H_2O.

are switched on to a small degree when the γ-genes are activated, but they are not fully activated until γ-chain synthesis diminishes at about 35 weeks of gestation. The rate of synthesis of the δ-chain is only $^1/_{140}$ that of the β-chain, due to differences in the promoter regions of the two genes. After birth, most cells produce predominantly α- and β-chains for the formation of HbA, the major adult hemoglobin (97%).

The synthesis of globin peptide chains occurs on polyribosomes in the cytoplasm of developing erythroblasts (Figure 6-6 ■). Globin chains are released from the polyribosomes and combine with heme molecules released from the mitochondria. The globin chains are folded to create a hydrophobic pocket near the exterior surface of the chain between the E and F helices. Heme is inserted into this hydrophobic pocket where it is readily accessible to oxygen. A newly formed α-chain-heme subunit and a non-α-chain-heme subunit combine spontaneously, facilitated by electrostatic attraction, to form a dimer (e.g., $\alpha\beta$). Charge differences exist among the non-α-globin chains. This promotes a hierarchy of affinity of these chains for the α-globin chains. The β-globin chain has the greatest affinity for α-globin chains followed by γ- and δ-chains. Then two dimers combine to form the tetrameric hemoglobin molecule (e.g., $\alpha_2\beta_2$). The protein alpha hemoglobin-stabilizing protein (AHSP) plays an important role in coordinating heme and globin assembly. AHSP binds free α-chains and stabilizes their structure, increasing their affinity for β-chains. This accelerates the formation of hemoglobin tetramers.[1]

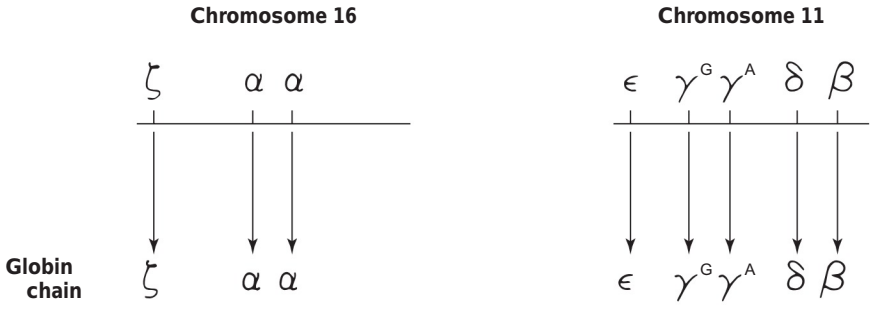


FIGURE 6-5 The genes for the globin chains are located on chromosomes 11 and 16. The ζ-chain appears to be the embryonic equivalent of the α-chain, both of which are located on chromosome 16. Note that the α-gene is duplicated. The other globin genes are located on chromosome 11.

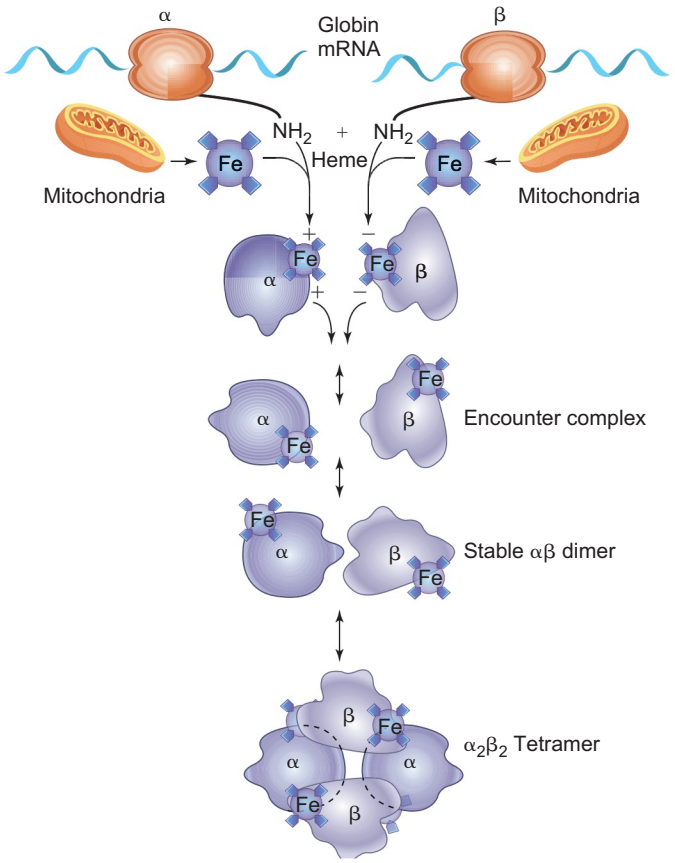


FIGURE 6-6 Assembly of hemoglobin. The α- and β-globin polypeptides are translated from their respective mRNAs. Upon heme binding, the protein folds into its native three-dimensional structure. The binding of α- and β-hemoglobin subunits to each other is facilitated by electrostatic attraction. An unstable intermediate encounter complex can rearrange to form the stable $\alpha\beta$ dimer. Two dimers combine to form the functional $\alpha_2\beta_2$ tetramer.

The heme is positioned between two histidines of the globin chain, the *proximal* (F8) and *distal* (E7) *histidines*. The proximal histidine is bonded with the heme iron. The iron is protected in the reduced ferrous state in this hydrophobic pocket. The exterior of the chain is hydrophilic, which makes the molecule soluble.

☑ CHECKPOINT 6-2

What globin chains are synthesized in the adult?

REGULATION OF HEMOGLOBIN SYNTHESIS

Balanced synthesis of globin chains and heme is important to the survival of the erythrocyte because hemoglobin tetramers are soluble, but individual components of hemoglobin such as unpaired globin chains, protoporphyrin, and iron are not. Normally, the production of α-globin subunits, non-α globin subunits, and heme are nearly equal. This indicates that tight regulatory mechanisms exist, controlling the production of hemoglobin. Hemoglobin synthesis is regulated by several mechanisms including:

- Activity and concentration of the erythroid enzyme 5-aminolevulinate synthase (ALAS2)
- Activity of porphobilinogen deaminase (PBGD)
- Concentration of iron
- Regulation of globin chain synthesis

The first step in the heme synthetic pathway, catalyzed by ALAS, is the rate-limiting step in heme synthesis and takes place in the mitochondria. ALAS is synthesized on ribosomes in the cytosol, and must be imported into the mitochondria to catalyze the reaction. This mitochondrial import can be inhibited by high concentrations of free heme.[2] Heme also inhibits uptake of iron from transferrin into the cell. When iron is scarce, the synthesis of ALAS is decreased.

Iron entering the developing erythroblast can be in either the pool available for metabolic processes (heme synthesis) or the storage pool (**ferritin** and **hemosiderin**). The amount of iron in these pools is regulated by proteins that control transcription and translation of proteins involved in heme synthesis and the formation of ferritin as well as transferrin receptors.[1–5] Iron metabolism is discussed in detail in Chapter 12.

The expression of globin genes occurs only in erythroid cells during a narrow period of differentiation. Synthesis of globin chains begins in the pronormoblast and continues until the reticulocyte loses remnants of mRNA. The rate of globin synthesis is governed primarily by the rate at which the DNA is transcribed to mRNA, but it is also modified by the processing of globin pre-mRNA to mRNA, the translation of mRNA to protein, and the stability of globin mRNA. The individual globin genes have separate promoter regions available for activation at variable times during embryonic and fetal development. In addition, the β-gene cluster has a locus control region (LCR), located upstream (5′) of the genes, which plays an important role in regulating the entire gene cluster. The α-gene cluster has a similar control region, the HS40, thought to have a similar function. Heme plays an important role in controlling the synthesis of globin chains. It stimulates globin synthesis by inactivating an inhibitor of translation. A slight excess of α-chain mRNA is produced, but the mRNA of β-chains is more efficiently translated, resulting in almost equal synthesis of α- and β-chains.

CASE STUDY *(continued from page 78)*

Jerry's doctor gave him iron supplements to take every day.

1. If Jerry is iron deficient, what is the effect of this deficient state on the synthesis of ALAS, transferrin receptor, and ferritin?
2. What was the rationale for giving Jerry the iron?

ONTOGENY OF HEMOGLOBIN

The type of hemoglobin is determined by its composition of globin chains (Table 6-2). Individual globin chains are expressed at different levels in developing erythroblasts of the human embryo, fetus, and adult. Some hemoglobins (Gower 1, Gower 2, Portland) occur only in the embryonic stage of development. HbF is the predominant hemoglobin in the fetus and newborn, and hemoglobin A is the predominant hemoglobin after 1 year of age.

The synthesis of different globin chains occurs in sequence depending on the developmental stage. This appears to be due to the sequential activation and then inactivation of transcription (i.e., "switching") among the α- and non-α-globin gene clusters. Globin gene expression is also affected by cellular, microenvironmental, and humoral influences that affect the proliferation and differentiation of stem cells. In vitro cultures of burst forming unit—erythroid (BFU-E; Chapter 4) from fetal liver, neonatal (umbilical cord) blood, and adult blood show HbF production from these three sources to decrease in concentration from fetal to neonatal to adult. The most important determinant of the switch from fetal to adult hemoglobin synthesis appears to be postconceptual age and is unaffected by the time of birth: Premature infants do not switch over to adult hemoglobin synthesis any earlier than they would if they had been carried full term. The developmental control of the perinatal switch from HbF to HbA synthesis appears to be intrinsic to the erythroid cell and is probably time controlled by a developmental clock.[6,7] The progenitor cells are gradually reprogrammed during the perinatal period, leading to a switching from γ-chain production to predominantly β-chain production. This can involve not only preferential stimulation of the β-globin gene but also active repression of the γ-globin gene.

Embryonic Hemoglobins

Embryonic erythropoiesis is associated with the production of the embryonic hemoglobins Gower 1, Gower 2, and Portland and apparently synthesized in succession as globin synthesis switches from $\zeta \rightarrow \alpha$ and from $\delta \rightarrow \gamma$ in the first trimester of gestation. Embryonic hemoglobins are made from the combination of pairs of embryonic globin chains, ζ and ϵ ($\zeta_2\,\varepsilon_2$) or embryonic chains in combination with α- and γ-chains ($\alpha_2\epsilon_2$, $\zeta_2\gamma_2$). These primitive hemoglobins are detectable during early hematopoiesis in the yolk sac and liver and are not usually detectable after the third month of gestation.

Fetal Hemoglobin

As embryonic erythropoiesis shifts to fetal erythropoiesis, hemoglobin F (HbF; $\alpha_2\gamma_2$) becomes the predominant hemoglobin formed during liver and bone marrow erythropoiesis in the fetus. HbF composes 90–95% of the total hemoglobin production in the fetus until ~34–36 weeks of gestation. At birth, the infant has 50–85% HbF.

Adult Hemoglobins

The fetal to adult shift in erythropoiesis reflects transcription of the β-globin chain. In adults, hemoglobin A (HbA; $\alpha_2\beta_2$) is the major hemoglobin. Although HbA is found as early as 9 weeks gestation, β-chain synthesis occurs at a low level until the third trimester of pregnancy. β-chain synthesis steadily increases from gestational week 30 onward but does not exceed γ-chain synthesis until after birth. After birth, the percentage of HbA continues to increase with the infant's age until normal adult levels (>95%) are reached by the end of the first year of life.

HbF production constitutes less than 2% of the total hemoglobin of adults. In normal adults, most if not all HbF is restricted to a few erythrocytes, referred to as *F cells*. F cells constitute 2–5% of adult RBCs, and from 13–25% of the hemoglobin within each F cell is HbF. The switch from HbF to HbA after birth is incomplete and in part reversible. For example, patients with hemoglobinopathies or severe anemia can have increased levels of HbF, often proportionate to the decrease in HbA. In bone marrow recovering from suppression and in some neoplastic hematologic diseases, HbF levels often rise.

HbA_2 ($\alpha_2\delta_2$) appears late in fetal life, composes < 1% of the total hemoglobin at birth, and reaches normal adult values (1.5–3.7%) after one year. The δ-gene locus is transcribed very inefficiently compared with the β-locus due to changes in the promoter region of the δ-gene that is recognized by erythroid-specific transcription factors (e.g., GATA-1). HbA_2 has a slightly higher oxygen affinity than HbA; otherwise, the two hemoglobins have similar or identical ligand binding curves, Bohr effect, and response to 2,3-BPG.

☑ CHECKPOINT 6-3

What are the names and globin composition of the embryonic, fetal, and adult hemoglobins?

Glycosylated Hemoglobin

Prolonged exposure of hemoglobin to chemically active compounds in the blood can result in nonenzymatic modification of hemoglobin. HbA_1 is a minor component of normal adult hemoglobin (HbA) that has been modified post-translationally in which a component has been added to (usually) the N terminus of the β-chain. Also known as "fast hemoglobin" or glycated hemoglobins, HbA_1 consists of three subgroups, HbA_{1a}, HbA_{1b}, and HbA_{1c}. The clinically most important subgroup of HbA_1 is HbA_{1c}, which is produced throughout the erythrocyte's life and is proportional to the concentration of blood glucose. Older erythrocytes typically contain more HbA_{1c} than younger erythrocytes, having been exposed to plasma glucose for a longer period of time. However, if young cells are exposed to extremely high concentrations of glucose (>400 mg/dL) for several hours, the concentration of HbA_{1c} increases with both concentration and time of exposure.

Measurement of HbA_{1c} is routinely used as an indicator of control of blood glucose levels in diabetics because it is proportional to the average blood glucose level over the previous 2–3 months. Average levels of HbA_{1c} are 7.5% in diabetics and 3.5% in normal individuals.

☑ CHECKPOINT 6-4

A patient has an anemia caused by a shortened RBC life span (hemolysis); how would this affect the HBA_{1c} measurement?

HEMOGLOBIN FUNCTION

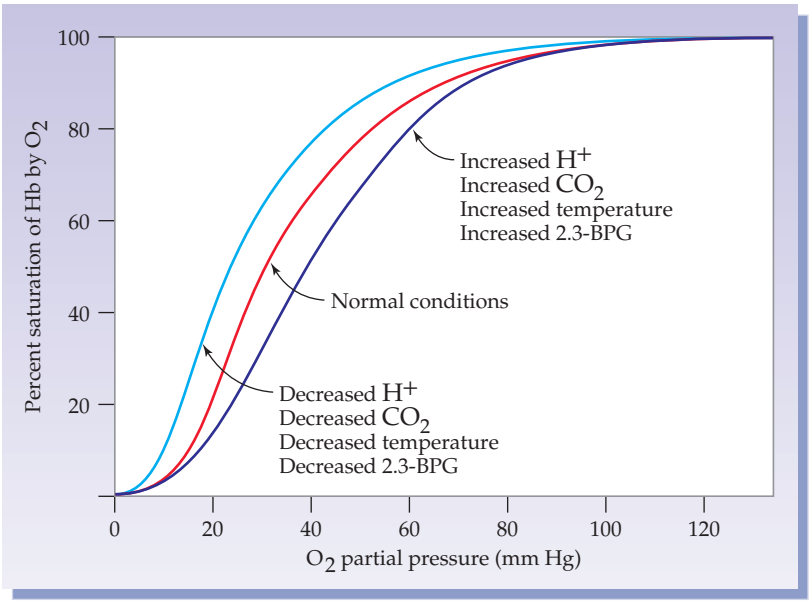
The function of hemoglobin is to transport and exchange respiratory gases. The air we breathe is a mixture of nitrogen, oxygen, water, and carbon dioxide. Each of the gases contributes to the atmospheric pressure (measured in mmHg) in proportion to its concentration. The partial pressure each gas exerts is referred to as P (e.g., PO_2) and determines the rate of diffusion of that gas across the alveolar-capillary membrane. Arterialized blood leaves the lungs with a PO_2 of 100 mmHg and a PCO_2 of 40 mmHg. In comparison, the PO_2 of interstitial fluid in tissues is about 40 mmHg, and the PCO_2 is about 45 mmHg. Thus, when blood reaches the tissues, oxygen diffuses out of the blood to the tissues and CO_2 diffuses into the blood from tissue. The amount of dissolved O_2 and CO_2 that the plasma can carry is limited. Most O_2 and CO_2 diffuse into the erythrocyte to be transported to tissue or lungs.

Oxygen Transport

Hemoglobin with bound oxygen is called **oxyhemoglobin**; hemoglobin without oxygen is called **deoxyhemoglobin**. The amount of oxygen bound to hemoglobin and released to tissues depends not only on the PO_2 and PCO_2 but also on the affinity of Hb for O_2. The ease with which hemoglobin binds and releases oxygen is known as **oxygen affinity**. Hemoglobin affinity for oxygen determines the proportion of oxygen released to the tissues or loaded onto the cell at a given oxygen pressure (PO_2). Increased oxygen affinity means that the hemoglobin has a high affinity for oxygen, will bind oxygen more avidly, and does not readily give it up; decreased oxygen affinity means the hemoglobin has a low affinity for oxygen and releases its oxygen more readily.

Oxyhemoglobin and deoxyhemoglobin have different three-dimensional configurations. In the unliganded or deoxy state, the tetramer is stabilized by intersubunit salt bridges and is described as being in the **tense (T) structure** or state. In oxyhemoglobin, the salt bridges are broken, and the molecule is described as being in the **relaxed (R) structure** or state. The change in conformation of hemoglobin (from T to R) occurs as a result of a coordinated series of changes in the quaternary structure of the tetramer as the subunits bind oxygen (see "The Allosteric Property of Hemoglobin"). The T configuration is a low oxygen-affinity conformation, and the R state is a high oxygen-affinity conformation.

Oxygen affinity of hemoglobin is usually expressed as the PO_2 at which 50% of the hemoglobin is saturated with oxygen (P_{50}). The P_{50} in humans is normally about 26 mmHg. If hemoglobin-oxygen saturation is plotted versus the partial pressure of oxygen (PO_2), a sigmoid-shaped (S-shaped) curve results. This is referred to as the *oxygen dissociation curve* (*ODC*) (Figure 6-7 ■). The shape of the curve reflects subunit interactions between the four subunits of hemoglobin (heme–heme interaction or cooperativity). Monomeric molecules such as myoglobin have a hyperbolic ODC indicating no cooperativity of oxygen binding. The sigmoid-shaped curve of hemoglobin dissociation indicates that the deoxyhemoglobin tetramer is slow to take up an O_2 molecule, but binding one molecule of O_2 to hemoglobin facilitates the binding of additional O_2. Thus, the "appetite" of hemoglobin for oxygen grows with the addition of each oxygen molecule.[8]



■ **FIGURE 6-7** The oxygen affinity of hemoglobin is depicted by the oxygen dissociation curve (ODC). The fractional saturation of hemoglobin (*y* axis) is plotted against the concentration of oxygen measured as the PO_2 (*x* axis). At a pH of 7.4 and an oxygen tension (PO_2) of 26 mmHg, hemoglobin is 50% saturated with oxygen (red line). The curve shifts in response to temperature, CO_2, O_2, 2,3-BPG concentration, and pH. When the curve shifts left (light blue line), there is increased affinity of Hb for O_2. When the curve shifts right (dark blue line), there is decreased affinity of Hb for O_2.

The shape of the curve has certain physiologic advantages. The "flattened" top of the *S* reflects the fact that >90% saturation of hemoglobin still occurs over a fairly broad range of PO_2. This enables us to survive and function in conditions of lower oxygen availability, such as living (or skiing) at high altitudes. Note that the steepest part of the curve occurs at oxygen tensions found in tissues. This allows the release of large amounts of oxygen from hemoglobin during the small physiologic changes in PO_2 encountered in the capillary beds of tissues. This is physiologically of great importance, for it allows the overall transfer of oxygen from the lungs to the tissues with relatively small changes in PO_2. The ODC shows that the oxygen saturation of hemoglobin drops from ~100% in the arteries to ~75% in the veins. This indicates that hemoglobin gives up about 25% of its oxygen to the tissues. When the curve is shifted to the right, the P_{50} is increased, indicating that the oxygen affinity has decreased. This results in the release of more oxygen to the tissues. When the curve is shifted to the left, the P_{50} is decreased, indicating that oxygen affinity has increased. In this case, less oxygen is released to the tissues.

CASE STUDY *(continued from page 83)*

Jerry was lethargic and pale and was having problems with activities of daily living.

3. Explain why Jerry could have these symptoms.

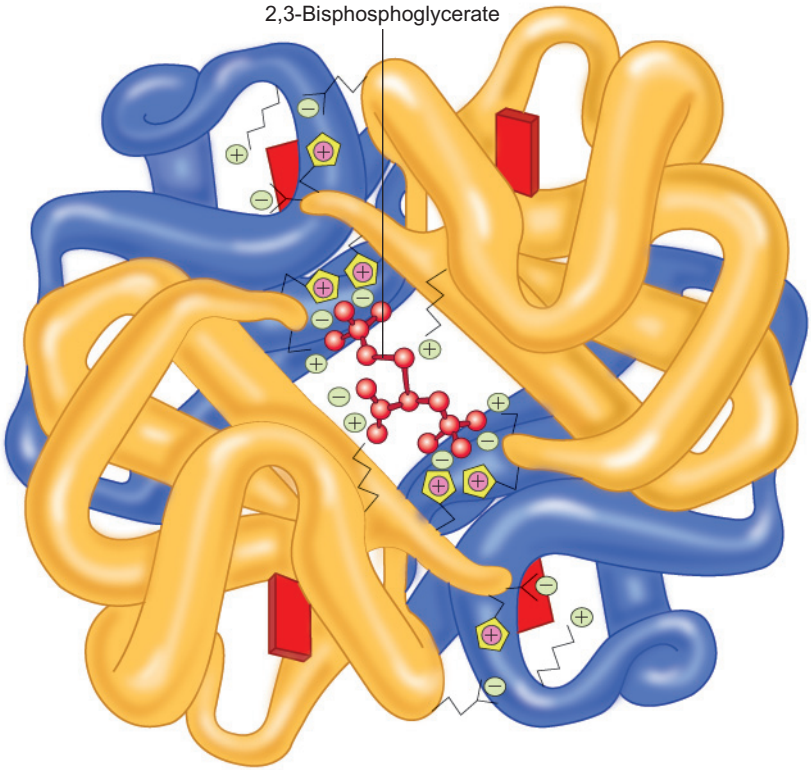
The Allosteric Property of Hemoglobin

The sigmoid shape of the ODC is primarily due to heme–heme interactions described below. However, the relative position of the curve (shifted right or left) is due to other variables.

Hemoglobin is an allosteric protein, meaning that its structure (conformation) and function are affected by other molecules. The primary allosteric regulator of hemoglobin is 2,3-bisphosphoglycerate (2,3-BPG; also referred to as 2,3-diphosphoglycerate [2,3-DPG]). A byproduct of the glycolytic pathway, 2,3-BPG, is present at almost equimolar amounts with hemoglobin in erythrocytes. In the presence of physiologic concentrations of 2,3-BPG, the P_{50} of hemoglobin is about 26 mmHg. In the absence of 2,3-BPG, the P_{50} of hemoglobin is 10 mmHg, indicating a very high oxygen affinity. Thus, in the absence of 2,3-BPG, little oxygen is released to the tissues.

Protons (H^+), CO_2, and organic phosphates (2,3-BPG) are all allosteric effectors of hemoglobin that preferentially bind to deoxyhemoglobin, forming salt bridges within and between the globin chains and stabilizing the deoxyhemoglobin (T) structure. The ratio in which 2,3-BPG binds to deoxyhemoglobin is 1:1. The binding site for 2,3-BPG is in a central cavity of the hemoglobin tetramer between the β-globin chains. It binds to positive charges on both β-chains, thereby crosslinking the chains and stabilizing the quaternary structure of deoxyhemoglobin (Figure 6-8 ■).

Hemoglobin also binds oxygen allosterically. Oxygen binds to hemoglobin in a 4:1 ratio because one molecule of O_2 binds to each



■ **FIGURE 6-8** 2,3-BPG binds in the central cavity of deoxyhemoglobin. This cavity is lined with positively charged groups on the beta chains that interact electrostatically with the negative charges on 2,3-BPG. The α-globin chains are in pink, the β-chains are in blue, and the heme prosthetic groups in red.

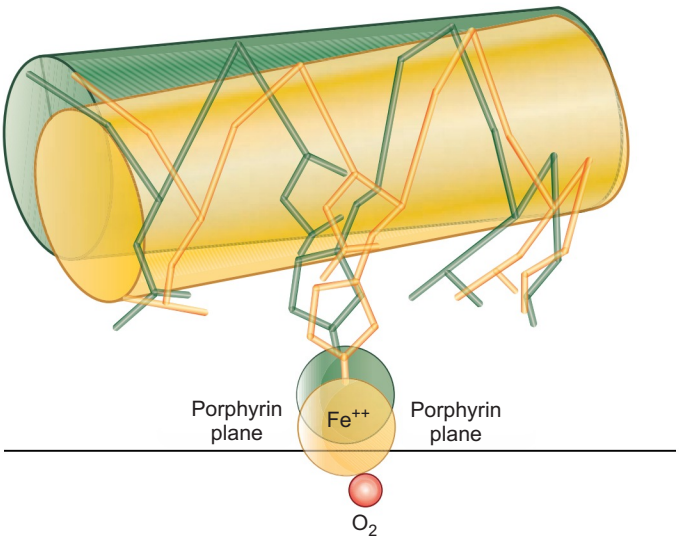
Source: Based on *Principles of Biochemistry, 4E* by H. R. Horton, L. A. Moran, K. G. Scrimgeour and M. D. Perry. Published by Pearson Education, Inc., © 2006.

of the four heme groups of the tetramer. The binding of oxygen by a hemoglobin molecule depends on the interaction of the four heme groups, referred to as *heme–heme interaction*. This interaction of the heme groups is the result of movements within the tetramer triggered by the uptake of a molecule of oxygen by one of the heme groups.

In the deoxygenated state, the heme iron is 0.4–0.6Å out of the plane of the porphyrin ring because the iron atom is too large to align within the plane. The iron is displaced toward the proximal histidine of the globin chain to which it is linked by a coordinate bond. Fully deoxygenated hemoglobin (T state) has a low oxygen affinity, and loading the first oxygen onto the tetramer does not occur easily. On binding of an oxygen molecule, the atomic diameter of iron becomes smaller due to changes in the distribution of electrons, and the iron moves into the plane of the porphyrin ring, pulling the histidine of the globin chain with it (Figure 6-9 ■). These small changes in the tertiary structure of the molecule near the heme group result in a large shift in the quaternary structure, altering the bonds and contacts between chains and weakening the intersubunit salt bridges. Likewise, loading a second O_2 onto the tetramer while it is still in the T conformation does not occur easily. However, the iron atom of the second heme is likewise shifted, further destabilizing the salt bridges. During the course of loading the third O_2 onto hemoglobin, the salt bridges are broken, and the hemoglobin molecule shifts from the T to the R configuration, pulling the β-chains together. Consequently, the size of the central cavity between the β-chains decreases, and 2,3-BPG is expelled. In the high oxygen–affinity R conformation, the third and fourth O_2 molecules are added easily. The structural changes within successive heme subunits facilitate binding the oxygen by the remaining heme subunits because fewer subunit crosslinks need to be broken to bind subsequent oxygen molecules. Thus, hemoglobin performs like a "mini-lung," changing shape as it takes up and releases O_2 to the tissue.

Oxygen interacts weakly with heme iron, and the two can dissociate easily. As O_2 is released by hemoglobin in the tissues, the heme pockets narrow and restrict entry of O_2, and the space between the β-chains widens and 2,3-BPG binds again in the central cavity. Thus, as 2,3-BPG concentration increases, the T configuration of hemoglobin is favored and the oxygen affinity decreases.

This cooperative binding of oxygen makes hemoglobin a very efficient oxygen transporter. Cooperativity ensures that once a hemoglobin tetramer begins to accept oxygen, it promptly is fully oxygenated. In the process of oxygen release to the tissues, the same general principle is followed. Individual hemoglobin molecules are generally either fully deoxygenated or fully oxygenated. Only a small portion of the molecules exists in a partially oxygenated state.



■ **FIGURE 6-9** Changes in the conformation of hemoglobin occur when the molecule takes up O_2. In the deoxyhemoglobin state, the heme iron of a hemoglobin subunit is below the porphyrin plane (green). On uptake of an O_2 molecule, the iron decreases in diameter and moves into the plane of the porphyrin ring, pulling the proximal histidine with it (yellow). The helix containing the histidine also shifts, disrupting ion pairs that link the subunits. 2,3-BPG is expelled, and the remaining subunits are able to combine with O_2 more readily.

Adjustments in Hemoglobin–Oxygen Affinity

Variations in environmental conditions or physiological demand for oxygen result in changes in erythrocyte and plasma parameters that directly affect hemoglobin–oxygen affinity. In particular, PO_2, pH (H^+), PCO_2, 2,3-BPG, and temperature affect hemoglobin–oxygen affinity (Table 6-3 ★).

Several physiologic mechanisms of oxygen delivery can be explained by the hemoglobin–2,3-BPG interaction. When going from sea level to high altitudes the body adapts to the decreased PO_2 by releasing more oxygen to the tissues. This adaptation is mediated by increases of 2,3-BPG in the erythrocyte, usually noted within 36 hours of ascent. EPO and erythrocyte mass also increase as a part of the body's adaptive mechanism to decreased PO_2 but this adaptation can take several days to improve tissue oxygenation.[9]

Fetal hemoglobin (HbF) has a higher oxygen affinity compared with adult hemoglobin, HbA. The γ-globin chain has a serine residue at the helical H21 position. In the β-globin chain, a histidine residue occupies this position. This change results in weak binding of 2,3-BPG

★ **TABLE 6-3** Factors That Affect Hemoglobin–Oxygen Affinity

Increase Affinity	Decrease Affinity
↑ O_2	↑ CO_2
↓ CO_2	↑ H^+
↓ H^+	↑ Temperature
↓ Temperature	↑ 2,3-BPG
↓ 2,3-BPG	

Key: ↑ = increased; ↓ = decreased

and increased oxygen affinity in HbF. The more efficient binding of 2,3-BPG to HbA facilitates the transfer of oxygen from the maternal (HbA) to the fetal (HbF) circulation.

Rapidly metabolizing tissue as occurs during exercise produces CO_2 and acid (H^+) as well as heat. These factors decrease the oxygen affinity of hemoglobin and promote the release of oxygen to the tissue. In the alveolar capillaries of the lungs, the high PO_2 and low PCO_2 drive off the CO_2 in the blood and reduce H^+ concentration, promoting the uptake of O_2 by hemoglobin (increasing oxygen affinity). Thus, PO_2, PCO_2, and H^+ facilitate the transport and exchange of respiratory gases.

The effect of pH on hemoglobin–oxygen affinity is known as the **Bohr effect**, an example of the acid–base equilibrium of hemoglobin that is one of the most important buffer systems of the body. A molecule of hemoglobin can accept H^+ when it releases a molecule of oxygen. Deoxyhemoglobin accepts and holds the H^+ better than oxyhemoglobin. In the tissues, the H^+ concentration is higher because of the presence of lactic acid and CO_2. When blood reaches the tissues, hemoglobin's affinity for oxygen is decreased by the high H^+ concentration, thereby permitting the more efficient unloading of oxygen at these sites.

$$Hb \cdot 4O_2 + 2H^+ \rightleftarrows Hb \cdot 2H^+ + 4O_2$$

Thus, proton binding facilitates O_2 release and helps minimize changes in the hydrogen ion concentration of the blood when tissue metabolism is releasing CO_2 and lactic acid. Up to 75% of the hemoglobin oxygen can be released if needed (as in strenuous exercise) as the erythrocytes pass through the capillaries.

☑ CHECKPOINT 6-5

What factors influence an increase in the amount of oxygen delivered to tissue during an aerobic workout?

Carbon Dioxide Transport

After diffusing into the blood from the tissues, carbon dioxide is carried to the lungs by three separate mechanisms: dissolution in the plasma, as HCO_3^- in solution, and binding to the N-terminal amino acids of hemoglobin (carbaminohemoglobin) (Table 6-4 ★; Figure 6-10 ■).

★ **TABLE 6-4** Carbon Dioxide Transport in Blood

Mechanism	Percent of Transportation
Dissolved in plasma	7
Formation of carbonic acid, H_2CO_3	70
Bound to Hb	23

Plasma Transport

A small amount of carbon dioxide is dissolved in the plasma and carried to the lungs. There it diffuses out of the plasma and is expired.

Carbonic Acid

Most of the carbon dioxide transported by the blood is in the form of bicarbonate ions (HCO_3^-), which are produced when carbon dioxide diffuses from the plasma into the erythrocyte. In the presence of the erythrocyte enzyme carbonic anhydrase (CA), CO_2 reacts with water to form carbonic acid (H_2CO_3).

$$H_2O + CO_2 \leftarrow CA \rightarrow H_2CO_3$$

Subsequently, hydrogen ion and bicarbonate are liberated from carbonic acid and the H^+ is accepted by deoxyhemoglobin:

$$H_2CO_3 \longleftarrow CA \longrightarrow \begin{matrix} HHb \\ \uparrow \\ H^+ + HCO_3^- \end{matrix}$$

The bicarbonate ions do not remain in the RBC because the cell can hold only a small amount of bicarbonate. Thus, the free bicarbonate diffuses out of the erythrocyte into the plasma. The cell cannot tolerate a loss in negative ions, so in exchange for the loss of bicarbonate, Cl^- diffuses into the cell from the plasma, a phenomenon called the **chloride shift**. This occurs via the anion exchange channel (band 3). The bicarbonate combines with Na^+ ($NaHCO_3$) in the plasma and is carried to the lungs where the PCO_2 is low. There the bicarbonate diffuses back into the erythrocyte, is rapidly converted back into CO_2 and H_2O, and is expired.

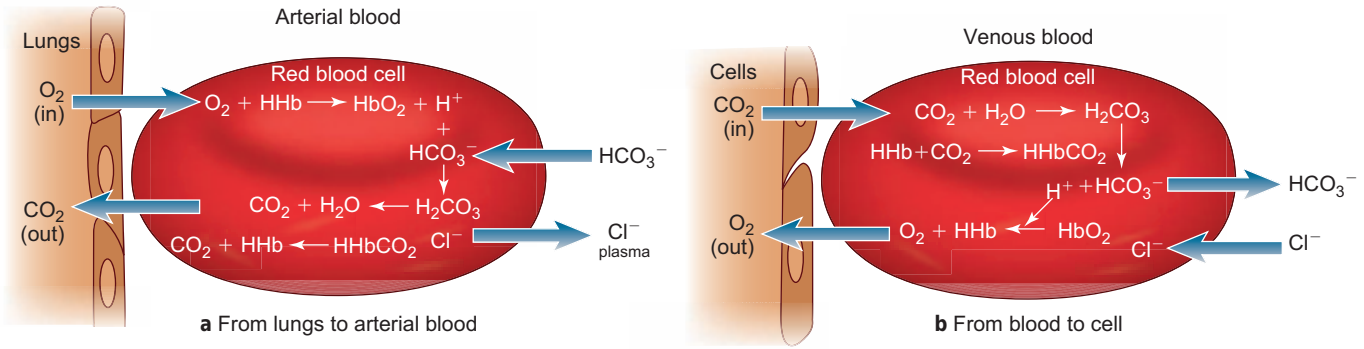
Hemoglobin Binding

Approximately 23% of the total CO_2 exchanged by the erythrocyte in respiration is through carbaminohemoglobin. Deoxyhemoglobin directly binds 0.4 moles of CO_2 per mole of hemoglobin. Carbon dioxide reacts with uncharged N-terminal amino groups of the four globin chains to form carbaminohemoglobin. At the lungs, the plasma PCO_2 decreases, and the CO_2 bound to hemoglobin is released and diffuses out of the erythrocyte to the plasma. It then is expired as it enters the alveolar air space.

CASE STUDY (continued from page 86)

After a week at home, Jerry called his doctor, who sent him back to the hospital where he was given 2 units of packed red cells. Within a day, he had more energy.

4. Explain why Jerry would have had more energy after the transfusions.



■ **FIGURE 6-10** Transport of oxygen and carbon dioxide in the erythrocyte is depicted. **(a)** In the lungs, O_2 and HCO_3^- enter the red blood cell. O_2 combines with Hb, releasing H^+. HCO_3^- combines with H^+ to form H_2CO_3, which dissociates into H_2O and CO_2, and CO_2 is expired. To maintain electrolyte balance, at the same time that HCO_3^- flows into the red blood cells, Cl^- flows out (the reverse chloride shift). The cell membrane anion-exchange protein (band 3) controls this ion exchange. Carbaminohemoglobin ($HHbCO_2$) releases CO_2 in the lungs (where the PCO_2 decreases) and is expired. The HHb releases the H^+ as Hb takes up oxygen. **(b)** CO_2 diffuses from the tissue into the venous blood and then into the erythrocyte. Within the erythrocyte, CO_2 reacts with water to form bicarbonic acid, H_2CO_3. The bicarbonic acid dissociates into a bicarbonate ion (HCO_3^-) and a proton (H^+). The HCO_3^- leaves the cell and enters the plasma. In exchange, chloride (Cl^-) from the plasma enters the erythrocyte (chloride shift). The proton facilitates the dissociation of oxygen from oxyhemoglobin (HbO_2) through the Bohr effect. When O_2 enters the tissues, the H^+ is taken up by deoxyhemoglobin.

Nitric Oxide and Hemoglobin

Nitric oxide (NO) is a critical component for the maintenance of blood vessel homeostasis. NO derived its name as the endothelium-derived relaxing factor (EDRF) because of its ability to relax smooth muscle and dilate blood vessels.[10] It is important in other aspects of normal vessel physiology as well as inhibition of platelet activation. NO is produced in the endothelium from arginine by the action of NO synthase. NO can diffuse from the plasma across the erythrocyte membrane where it is picked up by oxyhemoglobin. Reaction with oxyhemoglobin destroys the NO and forms methemoglobin and nitrate, a process known as *dioxygenation*.

$$HbO_2 + NO \rightarrow MetHb + NO_3^-$$

Reaction of NO and hemoglobin is limited because of hemoglobin compartmentalization in the erythrocyte, slow diffusion of NO across the RBC membrane, and the laminar blood flow that pushes the erythrocytes inward away from the vessel endothelium where the NO is concentrated.[11] The rate of reaction of NO with cell-free hemoglobin is increased by at least 1000 fold. This extracellular reaction is responsible for complications such as vasoconstriction and increase of blood pressure that are encountered when using artificial hemoglobin-based oxygen carriers in solution. The reaction also appears to be responsible for complications (e.g., high blood pressure) that accompany some hemolytic anemias such as sickle cell disease.

Artificial Oxygen Carriers

Efforts to reduce allogeneic blood transfusions and improve oxygen delivery to tissues have resulted in development of **artificial oxygen carriers (AOCs)**. Two groups of AOCs include hemoglobin-based oxygen carriers (HBOCs) in solution and perfluorocarbons (PFCs). The HBOCs consist of purified human or bovine hemoglobin or recombinant hemoglobin. The hemoglobin is altered chemically or genetically or is microencapsulated to decrease oxygen affinity and to prevent its breakdown into dimers that have significant nephrotic toxicity.[12] The oxygen dissociation curve of HBOCs is similar to that of native human blood. Adverse side effects of these AOCs are Hb-induced vasoconstriction and resulting hypertension. These side effects are related to NO scavenging and inactivation by the free hemoglobin as well as endothelin (a vasoconstrictor) release and sensitization of peripheral adrenergic receptors.[13] Because hemoglobin in solution imparts color to plasma, it might not be possible to perform laboratory tests based on colorimetric analysis of patients receiving this product because measurements could give erroneous results.

PFCs are fluorinated hydrocarbons with high gas-dissolving capacity. They do not mix in aqueous solution and must be emulsified. In contrast to HBOCs, a linear relationship between PO_2 and oxygen content in PFCs exists. This means that relatively high O_2 partial pressure is required to maximize delivery of O_2 by PFCs. The PFC droplets are taken up by the mononuclear phagocyte (MNP) system, broken down, bound to blood lipids, transported to the lungs, and exhaled.[13]

AOCs are not approved for use in the United States although HBOCs are approved for compassionate use.[13] Phase III trials are complete or in progress for HBOCs. No PFC has yet been approved for clinical use.[14]

HEMOGLOBIN CATABOLISM

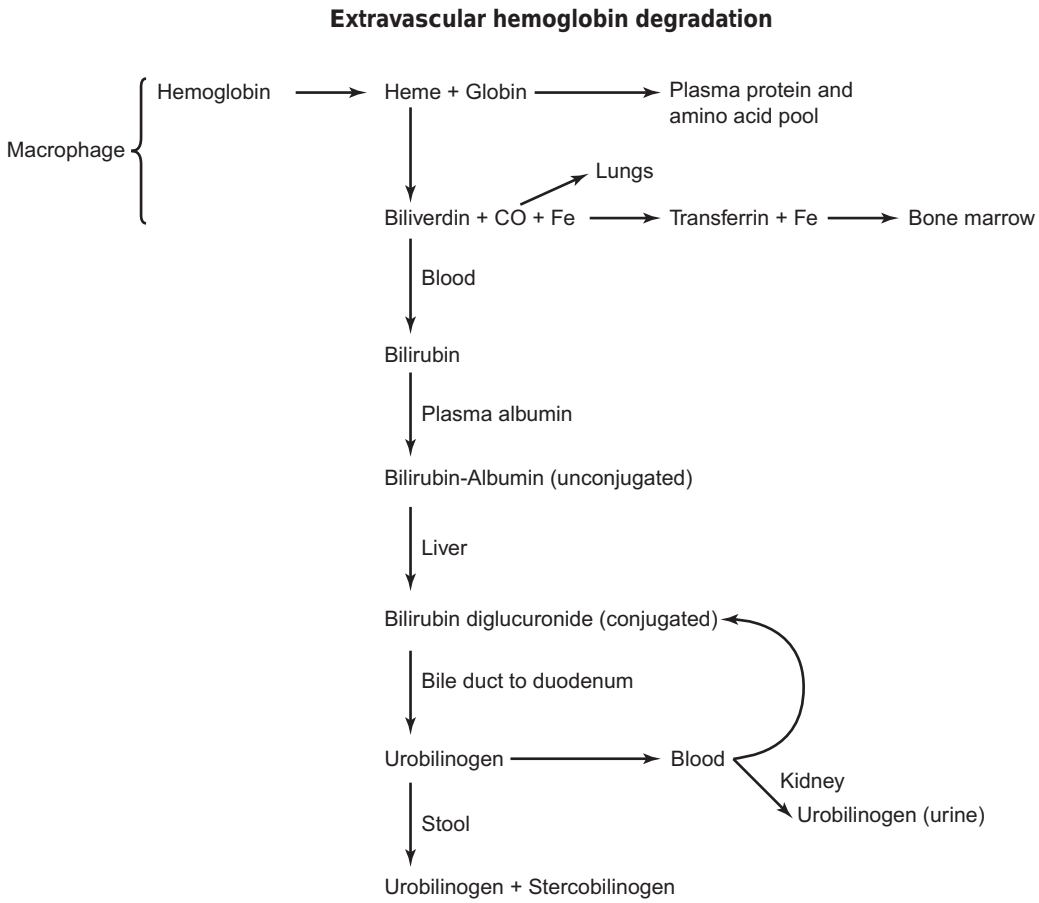
When the erythrocyte is removed from circulation by macrophages (extravascular hemolysis) or is lysed in the blood stream (intravascular hemolysis), hemoglobin is released and catabolized.

Extravascular Destruction

In extravascular hemolysis, erythrocyte removal by macrophages in the spleen, bone marrow, and liver conserves and recycles essential erythrocyte components such as amino acids and iron (Figure 6-11 ■). Most extravascular destruction of erythrocytes takes place in the macrophages of the spleen.

Within the macrophage, the hemoglobin molecule is broken down into heme, iron, and globin. Iron and globin (a polypeptide) are conserved and reused for new hemoglobin or other protein synthesis. Heme iron can be stored as ferritin or hemosiderin within the macrophage or released to the iron transport protein, transferrin, for delivery to developing normoblasts in the bone marrow. This endogenous iron exchange is responsible for about 80% of the iron passing through the transferrin pool. Thus, iron from the normal erythrocyte aging process is conserved and reutilized. The globin portion of the hemoglobin molecule is broken down and recycled into the amino acid pool.

Heme, the porphyrin ring, is further catabolized by the macrophage and eventually excreted in the feces. The α-methane bridge of the porphyrin ring is cleaved, producing a molecule of carbon monoxide and the linear tetrapyrrole biliverdin. Carbon monoxide is released to the blood stream, carried to the lungs, and expired. The biliverdin is rapidly reduced within the cell to bilirubin. Released from the macrophage, **bilirubin** is bound by plasma albumin and carried to the liver (this is called unconjugated or "indirect" bilirubin). Upon uptake by the liver, bilirubin is conjugated with two molecules of bilirubin glucuronide by the enzyme bilirubin UDP-glucuronyltransferase present in the endoplasmic reticulum of the hepatocyte. Once conjugated, bilirubin becomes polar and lipid insoluble.



■ **FIGURE 6-11** Most hemoglobin degradation occurs within the macrophages of the spleen. The globin and iron portions of the molecule are conserved and reutilized. Heme is reduced to bilirubin, eventually degraded to urobilinogen, and excreted in the feces. Thus, indirect indicators of erythrocyte destruction include the blood bilirubin level and urobilinogen concentration in the urine.

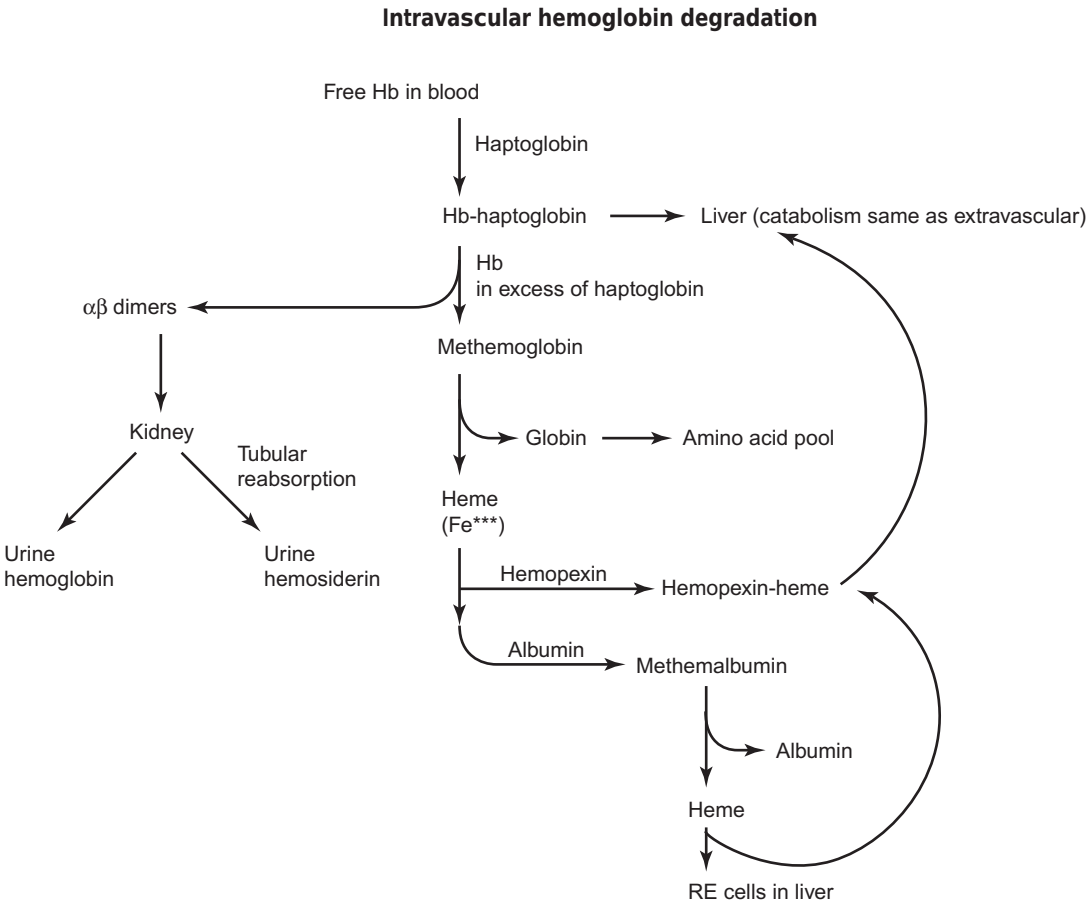
Bilirubin diglucuronide (called conjugated or "direct" bilirubin) is excreted into the bile, eventually reaching the intestinal tract where intestinal bacterial flora convert it into urobilinogen. Most urobilinogen is excreted in the feces where it is quickly oxidized to urobilin or stercobilin. However, 10–20% of the urobilinogen is reabsorbed from the gut back to the plasma. The reabsorbed urobilinogen is either excreted in urine or returned to the gut via an enterohepatic cycle. In liver disease, the enterohepatic cycle is impaired and an increased amount of urobilinogen is excreted in the urine.

Intravascular Destruction

The small amount of hemoglobin released into the peripheral blood circulation through intravascular erythrocyte breakdown undergoes dissociation into $\alpha\beta$ dimers, which are quickly bound to the plasma glycoprotein **haptoglobin** (Hp) in a 1:1 ratio (Figure 6-12 ■). Haptoglobin is an α2-globulin present in plasma at a concentration of 35–164 mg/dL (males) or 40–175 mg/dL (females). The haptoglobin–hemoglobin (HpHb) complex is too large to be filtered by the kidney, so haptoglobin carries hemoglobin dimers in the blood to the liver. Hepatocytes, which have haptoglobin receptors, take up the HpHb and process it in a manner similar to that of hemoglobin released by extravascular destruction.

The HpHb complex is cleared very rapidly from the bloodstream with a $T_{1/2}$ disappearance rate of 10–30 minutes. The haptoglobin concentration can be depleted very rapidly in acute hemolytic states because the liver is unable to maintain plasma haptoglobin levels. Haptoglobin, however, is an acute-phase reactant, and increased concentrations can be found in inflammatory, infectious, or neoplastic conditions. (An acute phase reactant is a protein whose plasma concentration increases in response to inflammation and serves a function in the immune response.) Therefore, patients with hemolytic anemia (anemia caused by increased destruction of erythrocytes) accompanied by an underlying infectious or inflammatory process can have normal haptoglobin levels.

When haptoglobin is depleted, as in severe hemolysis, free $\alpha\beta$ dimers can be filtered by the kidney and reabsorbed by the proximal tubular cells. $\alpha\beta$ dimers passing through the kidney in excess of the reabsorption capabilities of the tubular cells appear in the urine as free hemoglobin. Dimers reabsorbed by the tubular cells



■ **FIGURE 6-12** When the erythrocyte is destroyed within the vascular system, hemoglobin is released directly into the blood. Normally, the free hemoglobin quickly complexes with haptoglobin, and the complex is degraded in the liver. In severe hemolytic states, haptoglobin can become depleted, and free hemoglobin dimers are filtered by the kidney. In addition, with haptoglobin depletion, some hemoglobin is quickly oxidized to methemoglobin and bound to either hemopexin or albumin for eventual degradation in the liver.

are catabolized to bilirubin and iron, both of which can reenter the plasma pool. However, some iron remains in the tubular cell and is complexed to storage proteins forming ferritin and hemosiderin. Eventually, tubular cells loaded with iron are sloughed off and excreted in the urine (**hemosiderinuria**). The iron inclusions can be visualized with the Prussian blue stain. Thus, the presence hemosiderinuria is a sign of recent increased intravascular hemolysis.

Hemoglobin not excreted by the kidney or bound to haptoglobin is either cleared directly by hepatic uptake or oxidized to methemoglobin. Heme dissociates from methemoglobin and avidly binds to a β-globulin glycoprotein, **hemopexin**. Hemopexin is synthesized in the liver and combines with heme in a 1:1 ratio. The hemopexin–heme complex is cleared from the plasma slowly with a $T_{1/2}$ disappearance of 7–8 hours. When hemopexin becomes depleted, the dissociated oxidized heme combines with plasma albumin in a 1:1 ratio to form methemalbumin. Methemalbumin clearance by the liver is also very slow. In fact, methemalbumin may be only a temporary carrier for heme until more hemopexin or haptoglobin becomes available. Heme is transferred from methemalbumin to hemopexin for clearance by the liver as it becomes available. When present in large quantity, methemalbumin and hemopexin–heme complexes impart a brownish color to the plasma. The Schumm's test is designed to detect these abnormal compounds spectrophotometrically.

☑ CHECKPOINT 6-6

What lab tests would diagnose an increase in RBC destruction (i.e., hemolysis), and what would be the expected results?

ACQUIRED NONFUNCTIONAL HEMOGLOBINS

The acquired, nonfunctional hemoglobins are hemoglobins that have been altered post-translationally to produce molecules with compromised oxygen transport, thereby causing *hypoxia* and/or *cyanosis* (Table 6-5 ★). **Hypoxia** is a condition in which there is an inadequate amount of oxygen at the tissue level. (Hypoxemia is an inadequate amount of oxygen in the blood; arterial PO_2 <80 mmHg). **Cyanosis** refers to a bluish or slate-gray color of the skin due to the presence of more than 5 g/dL of deoxyhemoglobin in the blood.

Methemoglobin

Methemoglobin is hemoglobin with iron in the ferric (Fe^{+++}) state and is incapable of combining with oxygen. Methemoglobin not only decreases the oxygen-carrying capacity of blood but also results in an increase in oxygen affinity of the remaining normal hemoglobin. This results in an even higher deficit of O_2 delivery. Normally, methemoglobin composes < 3% of the total hemoglobin in adults.[15] At this concentration, the abnormal pigment is not harmful because the reduction in oxygen-carrying capacity of the blood is insignificant.

Clinically important methemoglobinemia can be due to the following (Table 6-6 ★):

1. Deficiencies of enzymes that reduce Fe^{+++}-hemoglobin to Fe^{++}-hemoglobin; of these, the most important, accounting for >60% of the reduction of methemoglobin, is NADH methemoglobin reductase (Table 6-7 ★).
2. Globin chain mutations that that stabilize heme iron in the Fe^{+++} state (hemoglobin M; Chapter 18). This structural variant of hemoglobin is characterized by amino acid substitutions in the globin chains near the heme pocket that stabilize the iron in the oxidized Fe^{+++} state.
3. Exposure to toxic substances that oxidize hemoglobin and overwhelm the normal reducing capacity of the cell. Increased levels of methemoglobin are formed when an individual is exposed to certain oxidizing chemicals or drugs. Even small amounts of these chemicals and drugs can cause oxidation of large amounts of hemoglobin. If the offending agent is removed, methemoglobinemia returns to normal levels within 24–48 hours.

Infants are more susceptible to methemoglobin production than adults because HbF is more readily converted to methemoglobin and because infants' erythrocytes are deficient in reducing enzymes. Exposure to certain drugs or chemicals that increase oxidation of hemoglobin or water high in nitrates can cause methemoglobinemia in infants. Color crayons containing aniline can cause methemoglobinemia if ingested.

Cyanosis develops when methemoglobin levels exceed 10% (>1.5 g/dL) hypoxia is produced at levels exceeding 30–40%. Toxic levels of methemoglobin can be reduced by medical treatment with methylene blue or ascorbic acid, which speeds up reduction by NADPH-reducing enzymes. The NADPH reductase system requires G6PD and therefore this method of treatment is not effective in patients with G6PD deficiency. In some cases of severe methemoglobinemia, exchange transfusions are helpful.

★ **TABLE 6-5** Abnormal Acquired Hemoglobins

Hemoglobin	Acquired Change	Abnormal Function	Lab Detection
Methemoglobin	Hb iron in ferric state	Cannot combine with oxygen	Demonstration of maximal absorption band at wave length of 630 nm; chocolate color blood
Sulfhemoglobin	Sulfur combined with hemoglobin	$^1/_{100}$ oxygen affinity of HbA	Absorption band at 620 nm
Carboxyhemoglobin	Carbon monoxide combined with hemoglobin	Affinity for carbon monoxide is 200 times higher than for oxygen	Absorption band at 541 nm

★ **TABLE 6-6** Differentiation of Types of Methemoglobinemia

Cause of Methemoglobinemia	Inherited/Acquired	Enzyme Activity	Hb Electrophoresis
Exposure to oxidants	Acquired	Normal	Normal
Decreased enzyme activity	Inherited	Decreased	Normal
Presence of hemoglobin M	Inherited	Normal	Abnormal

In the congenital methemoglobinemias, cyanosis is observed from birth, and methemoglobin levels reach 10–20%. Normal hemoglobin's oxygen affinity is increased in these children, resulting in increased erythropoiesis and subsequently higher than normal hemoglobin levels and erythrocytosis. Even in the homozygous state, individuals with HbM or defects in the reducing systems rarely have methemoglobin levels of >25% and are usually asymptomatic except for mild cyanosis. They do not usually require treatment. However, cyanosis can be improved by treatment with methylene blue or ascorbic acid. Laboratory diagnosis of methemoglobinemia involves demonstration of a maximum absorbance band at a wavelength of 630 nm at pH 7.0–7.4. The blood sample can be chocolate brown in color when compared with a normal blood specimen, and the color does not change to red upon exposure to oxygen.[15] Differentiation of acquired types from hereditary types of methemoglobin requires assay of NADH methemoglobin reductase and hemoglobin electrophoresis (Table 6-7). Enzyme activity is reduced only in hereditary NADH-methemoglobin reductase deficiency, and hemoglobin electrophoresis is abnormal only in HbM disease. Acquired methemoglobinemia shows normal enzyme activity and a normal electrophoresis pattern.

In the presence of methemoglobinemia, oxygen saturation obtained by a cutaneous pulse oximeter (fractional oxyhemoglobin, $FhbO_2$) can be lower than the oxygen saturation reported from a blood gas analysis. This is because $FhbO_2$ is calculated as the amount of oxyhemoglobin compared with the total hemoglobin (oxyhemoglobin, deoxyhemoglobin, methemoglobin, and other inactive hemoglobin forms) whereas oxygen saturation in a blood gas analysis is the amount of oxyhemoglobin compared with the total amount of hemoglobin able to combine with oxygen (oxyhemoglobin plus deoxyhemoglobin). $FhbO_2$ and oxygen saturation are the same if no abnormal hemoglobin is present.[16]

★ **TABLE 6-7** Erythrocyte Systems Responsible for Methemoglobin Reduction

Rank in Order of Decreasing Methemoglobin Reduction	System
First	NADH methemoglobin reductase (also known as *cytochrome b5 methemoglobin reductase, diaphorase I, DPNH-diaphorase, DPNH dehydrogenase I, NADH dehydrogenase, NADH methemoglobin-ferrocyanide reductase*)
Second	Ascorbic acid
Third	Glutathione
Fourth	NADPH methemoglobin reductase

Sulfhemoglobin

Sulfhemoglobin is a stable compound formed when a sulfur atom combines with the heme group of hemoglobin. The sulfur atom binds to a pyrrole carbon at the periphery of the porphyrin ring. Sulfuration of heme groups results in a drastically right-shifted oxygenation dissociation curve, which renders the heme groups ineffective for oxygen transport. This appears to be due to the fact that even half-sulfurated, half-oxygen–liganded tetramers exist in the T configuration (the low oxygen-affinity form) of hemoglobin. Although the heme iron is in the ferrous state, sulfhemoglobin binds to oxygen with an affinity only one-hundredth that of normal hemoglobin. Thus, oxygen delivery to the tissues can be compromised if there is an increase in this abnormal hemoglobin. The bright green sulfhemoglobin compound is so stable that the erythrocyte carries it until the cell is removed from circulation. Ascorbic acid or methylene blue cannot reduce it; however, sulfhemoglobin can combine with carbon monoxide to form carboxysulfhemoglobin. Normal levels of sulfhemoglobin do not exceed 2.2%. Cyanosis is produced at levels exceeding 3–4%.

Sulfhemoglobin has been associated with occupational exposure to sulfur compounds, environmental exposure to polluted air, and exposure to certain drugs. Sulfhemoglobinemia is formed during the oxidative denaturation of hemoglobin and can accompany methemoglobinemia, especially in certain drug- or chemical-induced hemoglobinopathies. Sulfhemoglobin is formed on exposure of blood to trinitroluene, acetanilid, phenacetin, and sulfonamides. It also is elevated in severe constipation and in bacteremia with *Clostridium welchii*. Diagnosis of sulfhemoglobinemia is made spectrophotometrically by demonstrating an absorption band at 620 nm. Confirmation testing is done by isoelectric focusing. This is the only abnormal hemoglobin pigment not measured by the cyanmethemoglobin method, which is used to measure hemoglobin concentration.

Carboxyhemoglobin

Carboxyhemoglobin is formed when hemoglobin is exposed to carbon monoxide. Hemoglobin's affinity for carbon monoxide is >200 times higher than its affinity for oxygen. Carboxyhemoglobin is incapable of transporting oxygen because CO occupies the same ligand-binding position as O_2. As is the case with methemoglobinemia, carboxyhemoglobin has a significant impact on oxygen delivery because it destroys the molecule's cooperativity. CO also has a

pronounced effect on the oxygen dissociation curve, shifting it to the left, resulting in increased affinity and a decreased release of O_2 by remaining normal hemoglobin molecules. High levels of carboxyhemoglobin impart a cherry red color to the blood and skin. However, high levels of it together with high levels of deoxyhemoglobin can give blood a purple-pink color.

Blood normally carries small amounts of carboxyhemoglobin formed from the carbon monoxide produced during heme catabolism. The level of carboxyhemoglobin varies depending on individuals' smoking habits and their environment. City dwellers have higher levels than country dwellers as a result of the carbon monoxide produced from automobiles and industrial pollutants in cities.

Acute carboxyhemoglobinemia causes irreversible tissue damage and death from anoxia. Chronic carboxyhemoglobinemia is characterized by increased oxygen affinity and polycythemia. In severe cases of carbon monoxide poisoning, patients can be treated in hyperbaric oxygen chambers.

Carboxyhemoglobin is commonly measured in whole blood by a spectrophotometric method. Sodium hydrosulfite reduces hemoglobin to deoxyhemoglobin, and the absorbances of the hemolysate are measured at 555 and 541 nm. Carboxyhemoglobin has a greater absorbance at 541 nm.

☑ CHECKPOINT 6-7

A 2-year-old child was found to have 15% methemoglobin by spectral absorbance at 630 nm. What tests would you suggest to help differentiate whether this is an inherited or acquired methemoglobinemia, and what results would you expect with each diagnosis?

Summary

Hemoglobin is the intracellular protein of erythrocytes responsible for transport of oxygen from the lungs to the tissues. A fine balance between production and destruction of erythrocytes serves to maintain a steady-state hemoglobin concentration.

Hemoglobin is a globular protein composed of four subunits. Each subunit contains a porphyrin ring with an iron molecule (heme) and a globin chain. The four globin chains are arranged in identical pairs, each composed of two different globin chains (e.g., $\alpha_2\beta_2$). Hemoglobin synthesis is controlled by iron concentration within the cell, synthesis and activity of the first enzyme in the heme synthetic pathway (ALAS), activity of PBGD, and globin chain synthesis.

The oxygen affinity of hemoglobin depends on PO_2, pH, PCO_2, 2,3-BPG, and temperature. Hemoglobin–oxygen affinity can be graphically depicted by the ODC. A curve that has shifted to the right reflects decreased oxygen affinity; when it has shifted to the left, oxygen affinity has increased. Increased CO_2, heat, and acid decrease oxygen affinity; high O_2 concentrations increase oxygen affinity.

Hemoglobin is an allosteric protein, which means that other molecules affect hemoglobin structure and function. In particular, the uptake of 2,3-BPG or oxygen can cause conformational changes in the molecule. The structure of deoxyhemoglobin is known as the *T structure* and that of oxyhemoglobin is known as the *R structure*.

When hemoglobin is exposed to oxidants or other compounds, the molecule can be altered, which can compromise its ability to carry oxygen. High concentrations of these abnormal hemoglobins can cause hypoxia and cyanosis, which can be detected by spectrophotometric methods.

Review Questions

Level I

1. Which of the following types of hemoglobin is the major component of adult hemoglobin? (Objective 4)
   A. HbA
   B. HbF
   C. HbA_2
   D. Hb Portland
2. One of the most important buffer systems of the body is the: (Objective 5)
   A. chloride shift
   B. Bohr effect
   C. heme–heme interaction
   D. ODC
3. When iron is depleted from the developing erythrocyte, the: (Objective 7)
   A. synthesis of heme is increased
   B. activity of ALAS is decreased
   C. formation of globin chains stops
   D. heme synthesis is not affected
4. When the H^+ concentration in blood increases, the oxygen affinity of hemoglobin: (Objective 3)
   A. increases
   B. is unaffected
   C. decreases
   D. cannot be measured

5. Which of the following is the correct molecular structure of hemoglobin? (Objective 1)
   A. four heme groups, two iron, two globin chains
   B. two heme groups, two iron, four globin chains
   C. two heme groups, four iron, four globin chains
   D. four heme groups, four iron, four globin chains
6. 2,3-BPG combines with which type of hemoglobin? (Objectives 3, 5)
   A. oxyhemoglobin
   B. relaxed structure of hemoglobin
   C. deoxyhemoglobin
   D. $\alpha\beta$ dimer
7. During exercise, the oxygen affinity of hemoglobin is: (Objective 3)
   A. increased in males but not females
   B. decreased due to production of heat and lactic acid
   C. unaffected in those who are physically fit
   D. affected only if the duration is more than 1 hour
8. Which of the following is considered a normal hemoglobin concentration in an adult male? (Objective 6)
   A. 11.0 g/dL
   B. 21.0 g/dL
   C. 15.0 g/dL
   D. 9.0 g/dL
9. Haptoglobin can become depleted in: (Objective 10)
   A. inflammatory conditions
   B. intravascular hemolysis
   C. infectious diseases
   D. kidney disease
10. A patient with an anemia due to increased extravascular hemolysis would likely present with which of the following lab results? (Objective 9)
    A. increased haptoglobin
    B. hemoglobinuria
    C. normal hemoglobin and hematocrit
    D. increased serum bilirubin

Level II

1. Which of the following hemoglobins is *not* found in the normal adult? (Objective 2)
   A. $\alpha_2\beta_2$
   B. $\alpha_2\gamma_2$
   C. $\alpha_2\delta_2$
   D. $\alpha_2\varepsilon_2$
2. Which of the following is the major hemoglobin in the newborn? (Objective 2)
   A. $\alpha_2\beta_2$
   B. $\alpha_2\gamma_2$
   C. $\alpha_2\delta_2$
   D. $\alpha_2\varepsilon_2$
3. A 2-year-old patient who had been cyanotic since birth was seen by a pediatrician. Blood was drawn for analysis of NADH methemoglobin reductase and results were normal. What follow-up test would you suggest to the physician? (Objective 6)
   A. hemoglobin electrophoresis
   B. bone marrow aspiration and examination
   C. haptoglobin and sulfhemoglobin determination
   D. glycosylated hemoglobin measurement by column chromatography
4. A 25-year-old male was found unconscious in a car with the motor running. Blood was drawn and sent to the chemistry lab for spectral analysis. The blood was cherry red in color. Which hemoglobin should be tested for? (Objective 6)
   A. sulfhemoglobin
   B. methemoglobin
   C. carboxyhemoglobin
   D. oxyhemoglobin
5. The oxygen dissociation curve in a case of chronic carboxyhemoglobin poisoning would show: (Objective 7)
   A. a shift to the right
   B. a shift to the left
   C. a normal curve
   D. decreased oxygen affinity
6. A college student from Louisiana vacationed in Colorado for spring break. He arrived at Keystone Resort on the first day. The second day, he was nauseated and had a headache. He went to the medical clinic at the resort and was told he had altitude sickness. The doctor told him to rest for another 24 hours before participating in any activities. What is the most likely reason he will overcome this condition in the next 24 hours? (Objective 4)
   A. His level of HbF will increase to help release more oxygen to the tissues.
   B. The amount of carboxyhemoglobin will decrease to normal levels.
   C. The levels of ATP in his blood will reach maximal levels.
   D. The level of 2,3-BPG will increase and, in turn, decrease oxygen affinity.

7. When iron in the cell is replete, the translation of ferritin mRNA is: (Objective 3)

   A. decreased

   B. increased

   C. unaffected

   D. variable

8. An aerobics instructor just finished an hour of instruction. Blood is drawn from her for a research study, and the oxygen dissociation is measured. What would you expect to find? (Objective 4)

   A. a shift to the left

   B. a shift to the right

   C. no shift

   D. an increased oxygen affinity

9. In the lungs, a hemoglobin molecule takes up two oxygen molecules. What effect will this have on the hemoglobin molecule? (Objectives 5, 8)

   A. It will increase oxygen affinity.

   B. It will narrow the heme pockets blocking entry of additional oxygen.

   C. The hemoglobin molecule will take on the tense structure.

   D. The center cavity will expand, and 2,3-BPG will enter.

10. An anemic patient has hemosiderinuria, increased serum bilirubin, and decreased haptoglobin. This is an indication that there is: (Objective 10)

    A. increased intravascular hemolysis

    B. decreased extravascular hemolysis

    C. hemolysis accompanied by renal disease

    D. a defect in the Rapoport-Leubering pathway

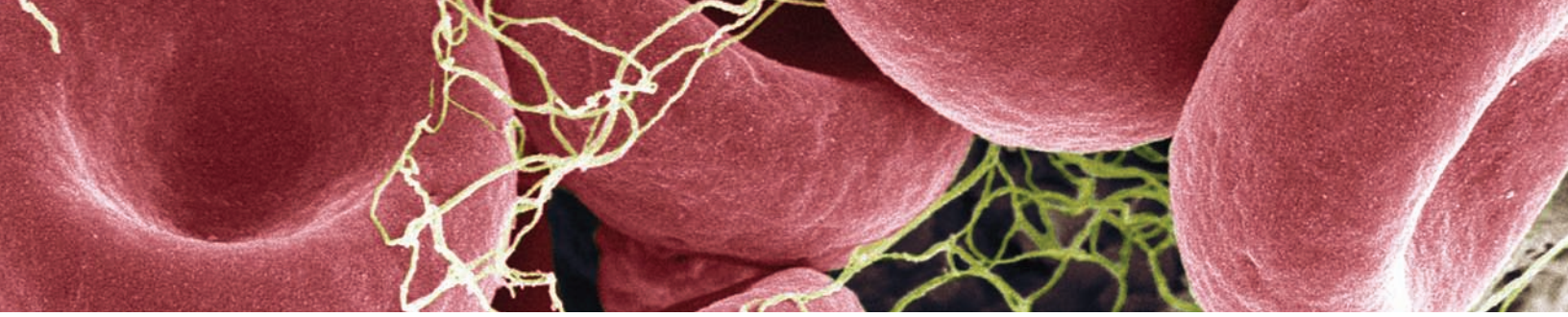
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Ferreira GC, Gong J. 5-aminolevulinate synthase and the first step of heme biosynthesis. *J Bioenerg Biomembr*. 1995;27:151–59.
2. Rouault TA. The role of iron regulatory proteins in mammalian iron homeostasis and disease. *Nature Chem Biol*. 2006;2(8):406–14.
3. Klausner RD, Rouault TA, Harford JB. Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell*. 1993;72:19–28.
4. Kawasaki N, Morimoto K, Tanimoto T et al. Control of hemoglobin synthesis in erythroid differentiating K562 cells. I. Role of iron in erythroid cell heme synthesis. *Arch Biochem Biophys*. 1996;328:289–94.
5. Kawasaki N, Morimoto K, Hayakawa T. Control of hemoglobin synthesis in erythroid differentiating K562 cells. II. Studies of iron mobilization in erythroid cells by high-performance liquid chromatography-electrochemical detection. *J Chromatogr B Biomed Sci Appl*. 1998;705:193–201.
6. Peschle C, Migliaccio AR, Migliaccio G et al. Regulation of Hb synthesis in ontogenesis and erythropoietic differentiation: in vitro studies on fetal liver, cord blood, normal adult blood or marrow, and blood from HPFH patients. In: Stamatoyannopoulos G, Nienhuis AW, eds. *Hemoglobins in Development and Differentiation*. New York: Alan R. Liss; 1980:359–71.
7. Papayannopoulou T, Nakamoto B, Agostinelli F et al. Fetal to adult hemopoietic cell transplantation in humans: Insights into hemoglobin switching. *Blood*. 1986;67:99–104.
8. Perutz MF. Molecular anatomy, physiology, and pathology of hemoglobin. In Stamatoyannopoulos G, Neinhuis AW, Leder P, Majerus PW, eds. *The Molecular Basis of Blood Diseases*. Philadelphia: WB Saunders; 1987.
9. Boning D, Maassen N, Jochum F et al. After effects of a high altitude expedition on blood. *Int J Sports Med*. 1997;18:179–85.
10. Schechter AN, Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med*. 2003;348(15):1483–85.
11. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler Thromb Vasc Biol*. 2006;26:697–705.
12. Henkel-Hanke T, Oleck M. Artificial oxygen carriers: A current view. *AANA J*. 2007;75(3):205–11.
13. Spahn DR. Blood substitutes artificial oxygen carriers: perfluorocarbon emulsions. *Crit Care*. 1999;3:R93–97.
14. Castro CI, Briceno JC. Perfluorocarbon-based oxygen carriers: Review of products and trials. *Artificial Organs*. 2010;34(8):622–34.
15. Benz EJ Jr, Ebert BL. Hemoglobin variants associated with hemolytic anemia, altered oxygen affinity, and methemoglobinemias. In Hoffman R, Benz EJ Jr, Silberstein LE et al. *Hematology: Basic Principles and Practice*. Philadelphia: Elsevier Churchill Livingstone, 2013. (accessed electronically)
16. Wentworth P, Roy M, Wilson B et al. Clinical pathology rounds: toxic methemoglobinemia in a 2-year-old child. *Lab Med*. 1999;30:311–15.



7 Granulocytes and Monocytes

KRISTIN LANDIS-PIWOWAR, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Identify terms associated with increases and decreases in granulocytes and monocytes.
2. Differentiate morphological features of the granulocyte and monocyte precursors found in the proliferative compartment of the bone marrow.
3. Describe the development, including distinguishing maturation and cell features, of the granulocytic and monocytic-macrophage cell lineages.
4. Describe and differentiate the morphologic and other distinguishing cell features of each of the granulocytes and monocytes found in the peripheral blood.
5. Explain the function of each type of granulocyte and monocyte found in the peripheral blood.
6. Summarize the process of neutrophil migration and phagocytosis.
7. List the adult reference intervals for the granulocytes and monocytes found in the peripheral blood.
8. Calculate absolute cell counts from data provided.
9. Differentiate and interpret absolute values and relative values of cell count data.
10. List causes/conditions that increase or decrease absolute numbers of individual granulocytes and monocytes found in the peripheral blood.
11. Compare and contrast pediatric and newborn reference intervals with adult reference intervals.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Summarize the kinetics of the granulocytic and monocytic-macrophage cell lineages.
2. Describe the processes that permit neutrophils to leave the peripheral blood circulation and move to a site of infection and propose how defects in these processes affect the body's defense mechanism.
3. Compare and contrast the immunologic features and functions of each of the granulocytes and monocytes found in the peripheral blood.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

4. Explain the physiological events that alter the number of circulating granulocytes and monocytes in the peripheral blood.
5. Correlate the laboratory data that pertain to granulocytes and monocytes with the clinical information for a patient.

Key Terms

Agranulocytosis
Azurophilic granule
Charcot-Leyden crystal
Chemokine
Chemotaxis
Circulating Pool (CP)
Cluster of differentiation (CD)
Degranulation
Diapedesis
Drumstick (Barr body)
Erythrophagocytosis
Granulocytosis
Leukocytosis
Leukopenia
Marginating pool (MP)
Monocyte-macrophage system
Mononuclear phagocyte (MNP) system
Neutropenia
Neutrophilia
Pathogen-associated molecular pattern (PAMP)
Pattern recognition receptor (PRR)
Phagocytosis
Polymorphonuclear

Background Basics

In addition to the basics from previous chapters, it is helpful to have a general understanding of immunology (immune system and function); biochemistry (proteins, carbohydrates, lipids); algebra; and the use of percentages, ratios, proportions, and the metric system.

To maximize your learning experience, you should review these concepts from previous chapters before starting this unit of study:

Level I

- Identify components of the cell and describe their function. (Chapter 2)
- Summarize the function of growth factors and the hierarchy of hematopoiesis. (Chapter 4)
- Describe the function of the hematopoietic organs. (Chapter 3)

Level II

- List the growth factors and identify their function in leukocyte differentiation and maturation. (Chapter 4)
- Describe the structure of the hematopoietic organs. (Chapter 3)

CASE STUDY

We will refer to this case study throughout the chapter.

Harry, a 30-year-old male in good physical condition, had a routine physical examination as a requirement for purchasing a life insurance policy. A CBC was ordered with the following results: Hb 15.5 g/dL (155 g/L), Hct 47% (0.47 L/L), RBC count 5.2 $\times$ 10^{12}/L, platelet count 175 $\times$ 10^{9}/L, and WBC count 12 $\times$ 10^{9}/L.

Consider how you could explain these results in a healthy male.

OVERVIEW

The terms *leukocyte* and *white blood cell (WBC)* are the synonymous names given to the nucleated blood cells that are involved in the defense against foreign pathogens or antigens. Leukocytes develop from the pluripotential hematopoietic stem cell in the bone marrow. In the presence of infection or inflammation, leukocytes can increase in number and can display morphologic changes. Thus, an important screening test for a wide variety of conditions is the leukocyte count, more commonly referred to as the *WBC count*. Leukocytes are classified as granulocytes (neutrophils, eosinophils, basophils), monocytes, and lymphocytes. This chapter is a study of the normal differentiation and maturation of granulocytes and the nongranulocytic monocyte. Each of these cells is discussed in terms of cell morphology, concentration in the peripheral blood, and function. Lymphocytes will be discussed in Chapter 8.

INTRODUCTION

With the exception of T lymphocytes, leukocyte precursors proliferate, differentiate, and mature in the bone marrow. Mature leukocytes are released into the peripheral blood where they circulate briefly until they move into the tissues in response to stimulation. They perform their function of host defense primarily in the tissues. The neutrophil, band neutrophil, eosinophil, basophil, monocyte, and lymphocyte are the leukocytes normally found in the peripheral blood of children and adults.

Leukocytes are nearly colorless in an unstained blood smear—hence, the term *leuko-*, meaning "white." The era of morphologic hematology began in 1877 with Paul Ehrlich's discovery of a triacidic stain that allowed for the differentiation of leukocytes on fixed blood smears.[1] Today, Wright stain, a Romanowsky-type stain, utilizes methylene blue and eosin to stain the cellular components of blood and bone marrow that are smeared on glass slides. Basic cellular elements react with the acidic dye (eosin), and acidic cellular elements react with the basic dye (methylene blue). The eosinophil contains large amounts of basic protein in its granules that react with the eosin dye—hence, the name *eosinophil*—whereas the basophil has granules that are acidic and react with the basic dye, methylene blue—hence, the name *basophil*. The neutrophil reacts with both acid and basic components of the stain, giving the cell cytoplasm a clear or tan to pinkish appearance with pink to violet stained granules. The nuclear DNA and cytoplasmic RNA of cells are acidic and pick up the basic stain, methylene blue. The eosinophil, basophil, and neutrophil are **polymorphonuclear** (their nuclei have many lobes) and because their cytoplasm contains many granules, they are classified as granulocytes. Monocytes are mononuclear cells and contain small numbers of fine granules in a bluish-gray cytoplasm.

William Hewson, the father of hematology, first observed leukocytes in the eighteenth century. In the nineteenth century, the studies of inflammation and bacterial infection intensified interest in leukocytes.[2] Many researchers studied the similarity of pus cells in areas of inflammation and the leukocytes of the blood. Ilya Metchnikov observed the presence of nucleated blood cells surrounding a thorn introduced beneath the skin of a larval starfish.[1]

Many of Ehrlich's observations and Metchnikov's experiments provided the groundwork for understanding the leukocytes as defenders against infection. Ehrlich recognized that variations in numbers of leukocytes accompanied specific pathologic conditions, such as eosinophilia in allergies, parasitic infections, and dermatitis as well as neutrophilia in bacterial infections.

Leukocytes function to fight infection by two separate but interrelated events: phagocytosis (innate immune response) and development of the adaptive immune response. Granulocytes and monocytes are the primary cells responsible for phagocytosis whereas monocytes and lymphocytes interact to produce an effective adaptive immune response (Chapter 8). Eosinophils and basophils interact in mediating allergic and hypersensitivity reactions.

LEUKOCYTE CONCENTRATION IN THE PERIPHERAL BLOOD

Leukocytes develop from pluripotential hematopoietic stem cells (HSCs) in the bone marrow. Upon specific hematopoietic growth factor stimulation, the stem cell proliferates and differentiates into the various types of leukocytes: granulocytes (neutrophils, eosinophils, basophils), monocytes, and lymphocytes. Once these cells have matured, they can be released into the peripheral blood or remain in the bone marrow storage pool until needed.

An individual's age and various physiologic and pathologic conditions predominantly affect the WBC count. The total WBC count is high at birth, ranging from 9–30 $\times$ 10^9/L. A few immature granulocytic cells (myelocytes, metamyelocytes) can be seen in the circulation during the first few days of life. However, immature leukocytes are not present in the peripheral blood after this age except in certain diseases. Within the first week after birth, the leukocyte count drops to 5–21 $\times$ 10^9/L. A gradual decline continues until the age of 8 years at which time the leukocyte concentration averages 8 $\times$ 10^9/L. Adult values average from 4.5–11.0 $\times$ 10^9/L, and generally do not decline with aging.[3]

In addition to age, physiologic and pathological events affect the concentrations of leukocytes. Pregnancy, time of day, and an individual's activity level affect the WBC concentration. Infections and immune-regulated responses cause significant changes in leukocytes. Many other pathologic disorders can also cause quantitative and/or qualitative changes in white cells. Considerable heterogeneity in leukocyte concentration has been found among racial, ethnic, and sex subgroups, suggesting the need for unique reference intervals for specific populations.[4] Thus, when WBC counts are evaluated, the patient's age, and possibly race/ethnicity and sex, provide useful information. It also is helpful to assess the accuracy of cell counts by correlating them with the patient's previous cell counts and clinical history. Additional testing, called *reflex testing*, can be indicated as a result of abnormalities in the WBC count. Changes associated with diseases and disorders will be discussed in subsequent chapters on leukocytes.

CASE STUDY *(continued from page 98)*

Harry's CBC results were Hb 15.5 g/dL (155 g/L), Hct 47% (0.47 L/L), RBC count 5.2 $\times$ 10^{12}/L, platelet count 175 $\times$ 10^9/L, and WBC count 12 $\times$ 10^9/L.

1. Are any of these results outside the reference interval? If so, which one(s)?
2. If this were a newborn, would you change your evaluation? If so, why?

An altered concentration of all leukocyte types or, more commonly, an alteration in one specific type of leukocyte can cause an increase or decrease in the total WBC count. For this reason, an abnormal total WBC count should be followed by a leukocyte differential count (commonly referred to as a *WBC differential*, or simply *diff*). A manual WBC differential is performed by enumerating each leukocyte type within a total of 100 leukocytes on a stained blood smear using a microscope. The differential results are reported as the percentage of each cell type counted. To accurately interpret whether an increase or decrease in cell types exists, however, it is necessary to calculate the absolute concentration using the results of the WBC count and the differential (relative concentration) in the following manner:

$$\text{Differential count (in decimal form)} \times \text{WBC count} \times (10^9/\text{L}) = \text{Absolute cell count} (\times 10^9/\text{L})$$

The application of this calculation is emphasized in the following example. Two different blood specimens from two different patients were found to have a relative neutrophil concentration of 85%. The total WBC count in one patient was 3 $\times$ 10^9/L and in the other was 9 $\times$ 10^9/L. The relative neutrophil concentration on both specimens appears elevated (reference interval is 40–80%); however, calculation of the absolute concentration (reference interval 1.8–7.0 $\times$ 10^9/L) shows that only one specimen has an absolute increase in neutrophils, whereas the other is within the reference interval:

$$0.85 \times (3 \times 10^9/\text{L}) = 2.6 \times 10^9/\text{L (within the reference interval)}$$
$$0.85 \times (9 \times 10^9/\text{L}) = 7.7 \times 10^9/\text{L (increased)}$$

Neutrophils comprise the largest portion of WBCs in peripheral blood followed by lymphocytes (Chapter 8), monocytes, eosinophils, and basophils, respectively. In an adult, neutrophils make up 40–80% of total leukocytes. At birth, the neutrophil concentration is about 50–60%; this level drops to ~30% by 4–6 months of age. After 4 years of age, the concentration of neutrophils gradually increases until adult values are reached at ~6 years of age (1.8–7.0 $\times$ 10^9/L). Most peripheral blood neutrophils are mature segmented forms. However, up to 5% of the less mature, nonsegmented forms, called *neutrophil bands*, can be seen in normal specimens. Most variations in the total WBC count are due to an increase or decrease in neutrophils.

Monocytes usually compose 2–10% (0.1–0.8 $\times$ 10^9/L) of circulating leukocytes. Occasionally, reactive lymphocytes (Chapter 8) resemble monocytes in morphology, posing classification difficulty even for the experienced hematologist.

Monocytes are functionally more similar to the granulocytes than to the nongranulocytic lymphocyte.

Peripheral blood eosinophil concentrations are maintained at 0–5% (up to 0.4 × 10^9/L) throughout life. It is possible that no eosinophils can be seen on a 100-cell differential. However, careful scanning of the entire smear should reveal an occasional eosinophil.

Basophils are the least plentiful cells in the peripheral blood, 0–1% (up to 0.2 × 10^9/L). It is common to find no basophils on a 100-cell differential. The finding of an absolute basophilia (>0.2 × 10^9/L), however, is very important because it can indicate the presence of a hematologic malignancy.

LEUKOCYTE SURFACE MARKERS

Leukocytes and other cells express a variety of molecules on their surfaces that can be used as markers to help identify the lineage of a cell as well as subsets within the lineage. These markers can be identified by reactions with specific monoclonal antibodies. A nomenclature system was developed to identify antibodies with similar characteristics using the term **cluster of differentiation (CD)** followed by a number. The CD designation is now used to identify the molecule recognized by the monoclonal antibody. In addition to using CD markers to identify cell lineage, some surface markers are used to identify stages of maturation as they are transiently expressed at a specific stage of development. Other markers are expressed only after the cell has been stimulated and thus can be used as a marker of cell activation. CD markers are very helpful in differentiating neoplastic hematologic disorders (Chapter 23) and can be identified by flow cytometry or cytochemical stains (Chapters 37, 40).

CASE STUDY (continued from page 99)

The WBC differential performed on the specimen from Harry had the following results:

Neutrophils	58%
Lymphocytes	32%
Monocytes	6%
Eosinophils	3%
Basophils	1%

3. Are any of the WBC concentrations outside the reference interval (relative or absolute)?

LEUKOCYTE FUNCTION

The primary function of leukocytes is to protect the host from infectious agents or pathogens by employing defense mechanisms called the *innate (natural)* and/or the *adaptive (acquired) immune systems*. The innate immune response (innate IR) is the body's first response to common classes of invading pathogens. When a pathogen enters the body, it must be recognized as foreign, or nonself, by soluble proteins (e.g., antibody or complement). The pathogen interacts with cell-surface receptors for IgG (FcγR) or complement (CR1, CR3) on leukocytes before the pathogen can be eliminated. The leukocyte receptors that participate in the innate IR are always available and do not require cell activation to be expressed.

Other pathogens can be eliminated without the step of recognition just described. This is accomplished when a pathogen contains certain structures shared by many different pathogens or common alterations that the pathogen makes to the body's cells. The shared structures or common cellular alterations are called **pathogen-associated molecular patterns (PAMPs)**. Examples include bacterial lipopolysaccharide, viral RNA, and bacterial DNA. Leukocytes are able to remove these pathogens by interaction with the leukocyte's surface receptors for PAMPs, referred to as **pattern recognition receptors (PRRs)**.[5] Once a pathogen has been recognized, effector cells can attack, engulf, and kill it. Neutrophils, monocytes, and macrophages play a major role in the innate immune system. The innate IR is rapid but limited.

The adaptive immune response (adaptive IR) is initiated in lymphoid tissue where pathogens encounter lymphocytes, the major cells involved in this response. This IR is slower to develop than the innate IR, but it provides long-lasting immunity (memory) against the pathogen with which it interacts. The adaptive IR will be discussed in more detail in Chapter 8.

In addition to its role in protection against infections, the cells of the innate immune system possess mechanisms to recognize the products of damaged and dead host cells, eliminating those cells and initiating tissue repair. These substances are called *damage-associated molecular patterns (DAMPs)* and include stress-associated heat shock proteins (HSPs), crystals, and nuclear proteins.[6]

NEUTROPHILS

Neutrophils are the most numerous leukocyte in the peripheral blood. They are easily identified on Romanowsky-stained peripheral blood smears as cells with a segmented nucleus and fine pink to lavender granules.

Differentiation, Maturation, and Morphology

Leukocytes develop from HSCs in the bone marrow. The common myeloid progenitor (CMP) cell gives rise to the committed precursor cells for the neutrophilic, eosinophilic, basophilic, and monocytic lineages, whereas the common lymphoid progenitor (CLP) cell gives rise to committed precursor cells for T, B, and natural killer (NK) lymphocytes[7] (Chapter 4). When lineage commitment has occurred, maturation begins. Myeloid and lymphoid cells go through unique maturation processes. The myeloid cells include the granulocytes and their precursor cells (granulocyte monocyte progenitor [GMP], colony-forming unit-granulocyte [CFU-G]), the eosinophilic and basophilic cells and their precursors (CFU-Eo, CFU-Ba) and the monocytic cells including monocytes and their precursors (GMP, CFU-M). The lymphoid cells include the lymphocytes and their precursors (CFU-T/NK, CFU-T, CFU-B).

Normally, the life span of the neutrophil is spent in three compartments: the bone marrow (site of proliferation, differentiation, and maturation), the peripheral blood (where they circulate for a few hours), and the tissues (where they perform their function of host defense).

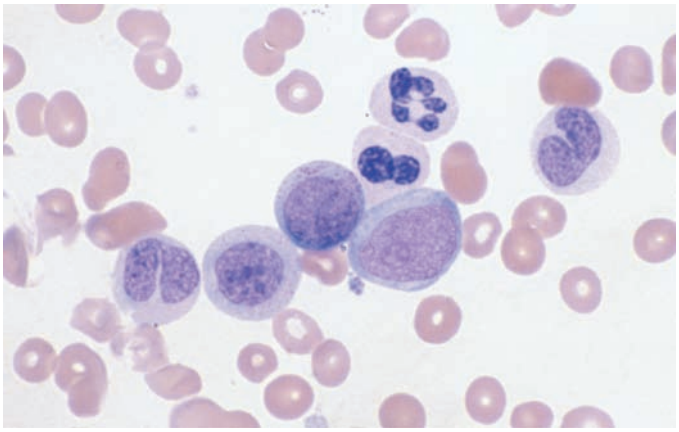
Neutrophilic production is primarily regulated by three cytokines, interleukin-3 (IL-3), granulocyte monocyte-colony-stimulating factor (GM-CSF), and granulocyte-colony-stimulating factor (G-CSF). GM-CSF and G-CSF also regulate survival and functional activity of mature neutrophils. The neutrophil undergoes six morphologically identifiable stages in the process of maturation. The stages from the first morphologically identifiable cell to the mature segmented neutrophil

include (1) myeloblast, (2) promyelocyte, (3) myelocyte, (4) metamyelocyte, (5) band or nonsegmented neutrophil, and (6) segmented neutrophil, also referred to as the *polymorphonuclear neutrophil (PMN)*.

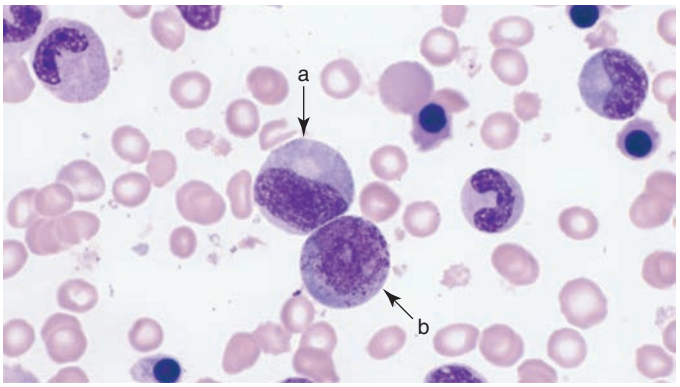
During the maturation process, progressive morphological changes occur in the nucleus. The nucleoli disappear, the chromatin condenses, and the once round nuclear mass indents and eventually segments. These nuclear changes are accompanied by distinct cytoplasmic changes. The scanty, agranular, basophilic cytoplasm of the earliest stage is gradually replaced by pink-to-tan-staining granular cytoplasm in the mature differentiated stage (Figures 7-1 ■ and 7-2 ■, Table 7-1 ★). The four subsets of granules/organelles (primary, secondary, secretory, tertiary) are produced at specific times during neutrophil development and contain specific molecules of physiologic importance. The biosynthesis of the granule content is primarily determined by activation or inhibition of transcription factors at certain time points during neutrophil development. Leukopoiesis is an amazing process that generates $1–5 \times 10^9$ cells per hour or 10^{11} cells per day.[7] However, the marrow has the capacity to significantly increase the neutrophil production over this baseline level in response to infectious or inflammatory stimuli. The morphology of the stages of maturation is discussed in the following sections.

Myeloblast

The myeloblast (Table 7-1, Figures 7-1 and 7-3 ■) is the earliest morphologically recognizable precursor of the myeloid lineage. The myeloblast size varies from 14–20 mcM (μm) in diameter, and it has a high nuclear to cytoplasmic (N:C) ratio. The nucleus is usually round or oval and contains a delicate, lacy, evenly stained chromatin. One to five nucleoli are visible. The small amount of cytoplasm is agranular, staining from deep blue to a lighter blue. A distinct unstained area adjacent to the nucleus representing the Golgi apparatus can be seen. Myeloblasts can stain faintly positive for peroxidase and esterase enzymes and for lipids (Sudan black B) although granules are not evident by light microscopy. Staining reactions with peroxidase and esterase help differentiate myeloblasts from monoblasts and lymphoblasts. CD markers also aid in identifying the lineage of blasts (Chapter 37). Myeloblast CD markers include CD33, CD13, CD38, and CD34.[8]



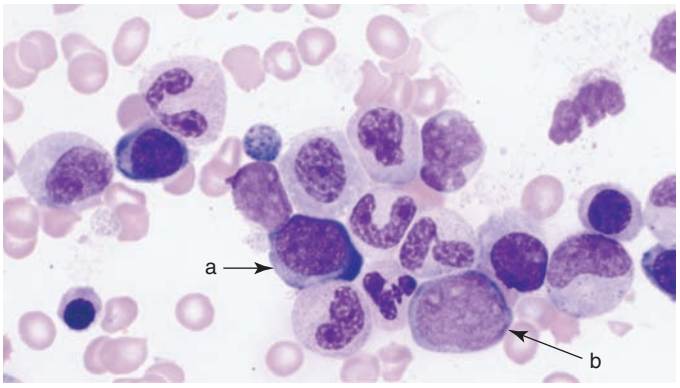
■ **FIGURE 7-1** Stages of neutrophil development. Compare the chromatin pattern of the nucleus and the cytoplasmic changes in the various stages. From left: a very early band, myelocyte, promyelocyte, myeloblast, and very early band; above the myeloblast are two segmented neutrophils (bone marrow; Wright-Giemsa stain; 1000× magnification).



■ **FIGURE 7-2** In the center are a myelocyte **(a)** and a promyelocyte **(b)**. Note the changes in the nucleus and cytoplasm. The myelocyte has a clear area next to the nucleus, which represents the Golgi apparatus. Note the azurophilic granules in the promyelocyte. Also present are two bands and in the top right corner a metamyelocyte. Orthochromatic normoblasts are present (bone marrow, Wright-Giemsa stain, 1000× magnification).

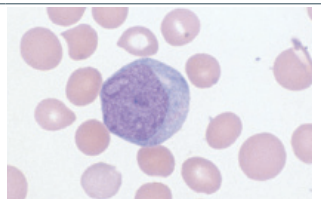
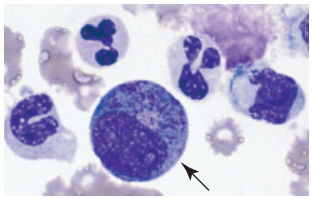
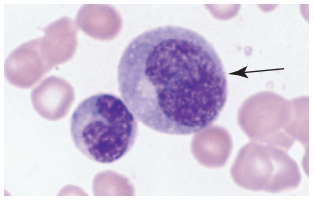
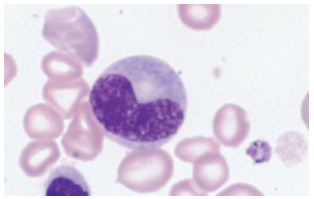
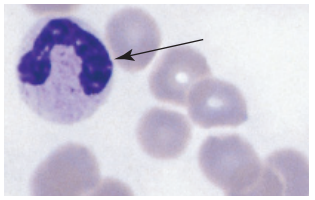
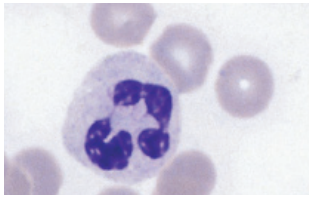
Promyelocyte

The promyelocyte/progranulocyte (Table 7-1, Figures 7-1 and 7-2) varies in size from 15–21 mcM. The nucleus is still quite large, and the N:C ratio is high. The nuclear chromatin structure, although coarser than that of the myeloblast, is still open and rather lacy, staining purple to dark blue. The color of the nucleus varies somewhat depending on the stain used, and several nucleoli can still be visible. The basophilic cytoplasm is similar to that of the myeloblast but is differentiated by the presence of prominent, reddish-purple primary granules, also called nonspecific or **azurophilic granules**, which are synthesized during this stage. The primary granules are



■ **FIGURE 7-3** **(a)** Indicates a pronormoblast and **(b)** indicates a myeloblast. Note that the myeloblast has more lacy, lighter-staining chromatin with distinct nucleoli and bluish cytoplasm whereas the pronormoblast chromatin is more smudged with indistinct nucleoli and very deep blue-purple cytoplasm. Also pictured are bands, metamyelocyte, myelocytes, basophilic normoblast, polychromatophilic normoblast, and orthochromatic normoblast (bone marrow, Wright-Giemsa stain; 1000× magnification).

★ TABLE 7-1 Characteristics of Cells in the Maturation Stages of the Neutrophil

Cell Stage (% in bone marrow)	Figure	Nucleus	Cytoplasm	N:C Ratio; Size (mcM)	Granules	CD Markers	Maturation Transit Time
Myeloblast (0.2–1.5)		Round or oval; delicate, lacy chromatin; nucleoli	Light blue	High; 14–20	Absent	CD13, CD33, CD34, CD38	~1 day
Promyelocyte (2–4)		Round or oval, chromatin lacy but more condensed than blast; nucleoli present	Deep blue	High but less than myeloblast; 15–21	Large, reddish-purple (azurophilic) primary or nonspecific granules	CD33, CD38	1–3 days
Myelocyte (8–16)		Round to oval; chromatin more condensed; nucleoli usually absent	Light blue, more mature shows tan to pink	Decreased from promyelocyte; 12–18	Small pinkish-red to specific granules; azurophilic granules; secretory vesicles		1–5 days
Metamyelocyte (9–25)		Chromatin condensed; stains dark purple; kidney bean shape to oval	Pinkish-tan	Decreased 10–18	Predominance of small pinkish-lavender specific granules; some azurophilic granules present; secretory vesicles		0.5–4 days
Band (nonsegmented) (9–15)		Chromatin condensed at ends of horseshoe-shaped nucleus; stains dark purple	Pink to tan to clear	Decreased	Abundant small, pinkish-lavender specific granules; some azurophilic granules present; secretory vesicles; tertiary granules		0.5–4 days
Segmented Neutrophil (polymorphonuclear)		Nucleus segmented into 2–4 lobes; chromatin condensed; stains deep purple/black	Pink or tan to clear	Decreased	As in band	CD15, CD16, CD11b/ CD18	1–5 days

surrounded by a phospholipid membrane and contain peroxidase and a number of antimicrobial compounds. See Table 7-2 ★ for a list of the contents of primary granules.

Myelocyte

The myelocyte (Table 7-1, Figures 7-1 through 7-3) varies in size from 12–18 mcM. The nucleus is reduced in size (as is the N:C ratio) due to nuclear chromatin condensation and appears more darkly stained than the chromatin of the promyelocyte. Nucleoli can be seen in the early myelocyte but are usually indistinct. The myelocyte nucleus can be round, oval, slightly flattened on one side, or slightly indented.[9] The clear light area next to the nucleus, representing the Golgi apparatus, can still be seen. The myelocyte goes through two to three cell divisions; this is the last stage of the maturation process in which the cell is capable of mitosis. The early myelocyte has a rather basophilic cytoplasm, whereas the later, more mature myelocyte, has a more tan to pink cytoplasm as the cell begins to lose cytoplasmic RNA.

The hallmark for the myelocyte stage is the appearance of specific or secondary granules. Synthesis of peroxidase-positive primary granules is halted, and the cell switches to synthesis of peroxidase-negative secondary granules. Secondary granules are detected first near the nucleus in the Golgi apparatus. This has sometimes been referred to as the *dawn of neutrophilia*. These neutrophilic secondary granules are small and sandlike with a pinkish-red to pinkish-lavender tint. Like the primary granules, a phospholipid membrane surrounds the secondary granules. Large primary azurophilic granules can still be apparent, but their concentration decreases with each successive cell division because their synthesis has ceased. Their ability to pick up stain also decreases with successive mitotic divisions. See Table 7-2 for a partial list of secondary granule contents.[9]

Secretory vesicles are scattered throughout the cytoplasm of myelocytes, metamyelocytes, band neutrophils, and segmented neutrophils[10] (Table 7-2). Secretory vesicles are formed by endocytosis in the later stages of neutrophil maturation and contain plasma proteins including albumin. When neutrophils are stimulated, the cytoplasmic secretory vesicles fuse with the plasma membrane to increase the neutrophil surface membrane and expression of adhesion and chemotactic receptors.

Metamyelocyte

The metamyelocyte (Table 7-1, Figures 7-1 and 7-3) varies in size from 10–18 mcM in diameter and is not capable of cell division. Nuclear indentation that gives the nucleus a kidney bean shape can be a characteristic that differentiates a metamyelocyte from a myelocyte, but nuclear shape is variable and is not the most reliable identifying feature. Care should be taken to review other cellular features such as the degree of the chromatin clumping, color of the cytoplasm, predominant granules present, and the cell size. The nuclear chromatin is coarse and clumped and stains dark purple. Nucleoli are not visible. The cytoplasm has a predominance of secondary and secretory granules. The ratio of secondary to primary granules is ~2:1. The metamyelocyte's cytoplasm resembles the color of the cytoplasm of a fully mature neutrophil (pinkish-tan). Tertiary or gelatinase-containing granules are synthesized mainly during the metamyelocyte and band neutrophil stages.[9]

Band Neutrophil

The band neutrophil, also called *nonsegmented neutrophil* or *stab*, is slightly smaller in diameter than the metamyelocyte. The metamyelocyte becomes a band when the indentation of the nucleus is more than half the diameter of the hypothetical round nucleus (Table 7-1 and Figure 7-1). The indentation gives the nucleus a horseshoe shape. The chromatin displays increased condensation at either end of the nucleus. The cytoplasm appears pink to tan, resembling both the previous stage and the fully mature segmented forms. The band neutrophil is the first stage that normally is found in the peripheral blood. All four types of granules (primary, secondary, secretory, tertiary) can be found at this stage, but primary granules are not usually differentiated with Wright stain in band neutrophils.

Segmented Neutrophil

Although similar in size to the band form, the neutrophil, or PMN, is recognized, as its name implies, by a segmented nucleus with two or more lobes connected by a thin nuclear filament (Table 7-1, Figure 7-1). The chromatin is condensed and stains a deep purple black. Most neutrophils have three or four nuclear lobes, but a range of two to five lobes is possible. Fewer than three lobes are considered *hyposegmented*. A cell with more than five lobes is considered abnormal and referred to as a *hypersegmented neutrophil*. Observing three or more five-lobed neutrophils in a 100-cell differential is usually considered pathologic (megaloblastic anemia; Chapter 15). Nuclear lobes are often touching or superimposed on one another, sometimes making it difficult to differentiate the cell as a band or PMN.

Individual laboratories and agencies such as the Clinical and Laboratory Standards Institute (CLSI) have outlined criteria for differentiating bands from PMNs in manual differentials.[11] A *band* is defined as having a nucleus with a connecting strip or isthmus with parallel sides and having width enough to reveal two distinct margins with nuclear

★ **TABLE 7-2** Neutrophil Granule Contents

Primary Granules	Secondary Granules	Secretory Vesicles	Tertiary Granules
Myeloperoxidase	Lactoferrin	Alkaline phosphatase	Gelatinase
Lysozyme	Lysozyme	Complement receptor 1	Lysozyme
Cathepsin G, B, and D	Histaminase	Cytochrome b_{558}	
Defensins (group of cationic proteins)	Collagenase		
Bactericidal permeability increasing protein (BPI)	Gelatinase		
Esterase N	Heparinase		
Elastase			

chromatin material visible between the margins. If a margin of a given lobe can be traced as a definite and continuing line from one side across the isthmus to the other side, a filament is assumed to be present although it is not visible. If a laboratory professional is not sure whether a neutrophil is a band form or a segmented form, it is arbitrarily classified as a segmented neutrophil. From a traditional clinical viewpoint, determining whether young forms of neutrophils (band forms and younger) are increased has been useful.[9] However, differentials performed by automated hematology instruments do not differentiate between band and segmented neutrophils. Band neutrophils are fully functional phagocytes and often are included with the total neutrophil count.[11]

The cytoplasm of the mature PMN stains a pink or tan to clear color. It contains many secondary and tertiary granules and secretory vesicles. Primary granules are present, but because of their loss of staining quality, might not be readily evident. The ratio of secondary to primary granules remains ~2:1.

Neutrophilic granules contain protein, lipids, and carbohydrates. Many of the proteins (enzymes) have already been discussed. About one-third of the lipids in neutrophils consist of phospholipids. Much of the phospholipid is present in the plasma membrane or membranes of the various granules. Cholesterol and triglycerides constitute most of the nonphospholipid neutrophil lipid. Although cytoplasmic nonmembrane lipid bodies can also be found in neutrophils, their role in cell function is unclear. Lipid material is likewise found in neutrophilic precursors and a cytochemical stain for lipids, Sudan black B, is used to differentiate myeloid precursors from lymphoid precursors (Chapter 23). Carbohydrate in the form of glycogen is also found in neutrophils and some myeloid precursors. Neutrophils utilize glycogen to obtain energy by glycolysis when required to function in hypoxic conditions (e.g., an abscess site). The periodic acid-Schiff (PAS) stain is used to detect glycogen in cells. CD markers on the neutrophil include CD13, CD15, CD16, CD11b/CD18, and CD33.[8]

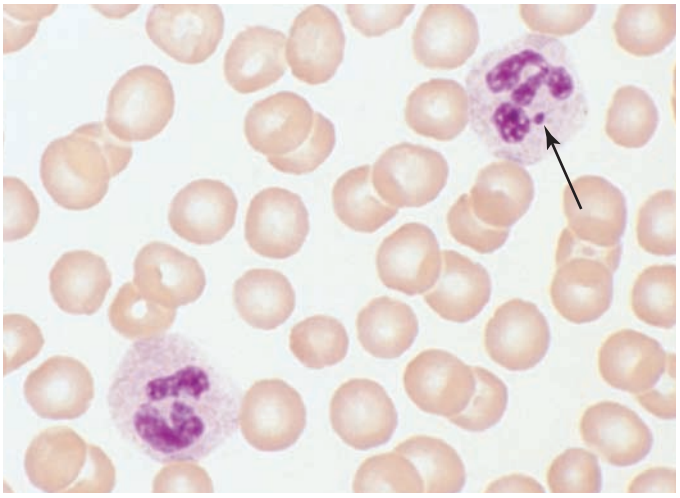
In normal females with two X chromosomes or males with XXY chromosomes (Klinefelter syndrome), one X chromosome is randomly inactivated in each somatic cell of the embryo and remains inactive in all daughter cells produced from that cell. The inactive X chromosome appears as an appendage of the neutrophil nucleus and is called a **drumstick (Barr body)** or an *X chromatin body* (Figure 7-4 ■). The number of chromatin bodies detected in the neutrophil is one less than the number of X chromosomes present; however, chromatin bodies are not visible in every neutrophil. The X chromatin bodies can be identified in 2–3% of the circulating PMNs of 46, XX females, and Klinefelter males (47, XXY).[9]

☑ CHECKPOINT 7-1

An adult patient's peripheral blood smear revealed many myelocytes, metamyelocytes, and band forms of neutrophils. Is this a normal finding?

Distribution, Concentration, and Kinetics

The kinetics of a group of cells—their production, distribution, and destruction—also is described as the *cell turnover rate*. For the neutrophil, kinetics follows the movement of the cell through a series of interconnected compartments (the marrow, blood, tissues).



■ **FIGURE 7-4** The segmented neutrophil on the right has an X chromatin body (arrow) (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Bone Marrow

Neutrophils in the bone marrow are derived from the stem cell pool and can be divided into two pools: the mitotic pool and the postmitotic pool (Figure 7-5 ■). The *mitotic pool*, also called the *proliferating pool*, includes cells capable of DNA synthesis: myeloblasts, promyelocytes, and myelocytes. Cells spend about 3–6 days in this proliferating pool and undergo four to five cell divisions. Although two to three of these divisions occur in the myelocyte stage, the number of cell divisions at each stage is variable. The postmitotic pool, also known as the *maturation and storage pool*, includes metamyelocytes, bands, and segmented neutrophils. Cells spend about 5–7 days in this compartment before they are released to the peripheral blood. However, during infections, the myelocyte-to-blood transit time can be as short as 2 days. The number of cells in the postmitotic storage pool is almost three times that of the mitotic pool.[10]

The largest compartment of neutrophils is found within the bone marrow and is referred to as the *mature neutrophil reserve*. The number of neutrophils circulating in the peripheral blood, the blood compartment, is about one-third the size of the bone marrow compartment. Once precursor cells have matured in the bone marrow, they are released into the peripheral blood (Chapter 4). Normally, the input of neutrophils from the bone marrow to the peripheral blood equals the output of neutrophils from the blood to the tissues, maintaining a relative steady-state blood concentration. However, when the demand for neutrophils increases, as in infectious states, the neutrophil concentration in the peripheral blood can increase quickly by their release from the bone marrow storage (reserve) pool. Depending on the strength and duration of the stimulus, the marrow myeloid precursor cells (GMP, CFU-G) also can be induced to proliferate and differentiate to form additional neutrophils. The transit time between development in the bone marrow and release to the peripheral blood can be decreased as a result of several mechanisms: (1) acceleration of maturation, (2) skipped cellular divisions, and (3) early release of cells from the marrow.

The mechanisms regulating the production and release of neutrophils from the bone marrow to the peripheral blood are not completely understood but likely include a feedback loop between the

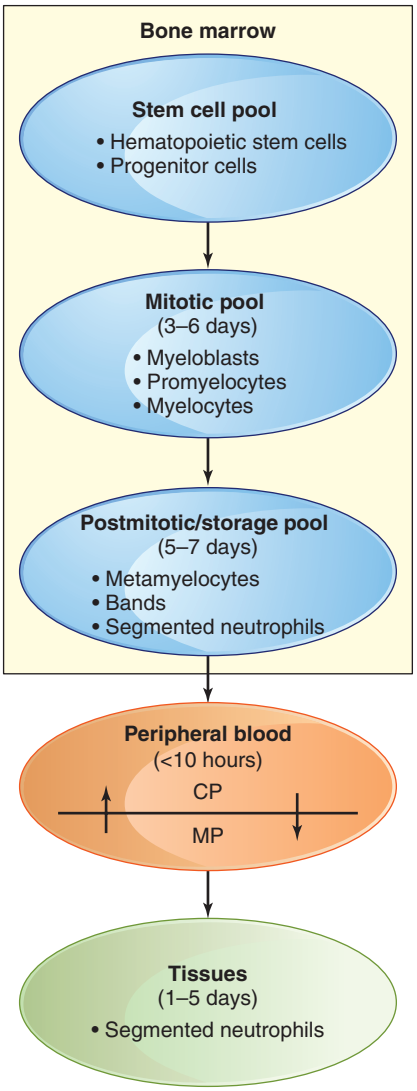


FIGURE 7-5 Neutrophils are produced from stem cells in the bone marrow and spend about 1–2 weeks in this maturation compartment. Most neutrophils are released to the peripheral blood as segmented forms. When the demand for these cells is increased, more immature forms can be released. One-half of the neutrophils in the peripheral blood are in the marginating pool (MP); the other half are in the circulating pool (CP). Neutrophils spend <10 hours in the blood before marginating and exiting randomly to tissue.

circulating neutrophils and the bone marrow. In normal conditions, this mediator is likely G-CSF, produced by marrow stromal cells and macrophages. Inflammatory cytokines such as IL-1 and tumor necrosis factor (TNF) are important in causing an increase in the neutrophil concentration in pathologic conditions by inducing the macrophage to increase the release of G-CSF and GM-CSF. The vascular endothelial cells (VECs) that form the inner lining of blood vessels also generate cytokines that govern activation and recruitment of leukocytes. Endothelial cells can be important in recruiting neutrophils in the earliest phases of inflammation and injury.

The release mechanism of the bone marrow storage pool is selective in normal, steady-state kinetics, releasing only segmented neutrophils and a few band neutrophils. The mechanisms controlling this regulated release are not fully understood. The release is partially regulated by the small pore size in the vascular endothelium lining the bone marrow sinusoids and by the mature segmented neutrophil's ability to deform enough to squeeze through the narrow opening. Immature cells are larger and less deformable and cannot penetrate the small pores; however, when an increased demand for neutrophils exists, a higher proportion of less mature neutrophils is released into the peripheral blood. Glucocorticoids, endotoxin (bacterial lipopolysaccharide), and G-CSF can increase neutrophil release from the marrow.

Peripheral Blood

Neutrophils are released from the bone marrow to the peripheral blood, but not all neutrophils are circulating freely at the same time. About half the *total blood neutrophil pool* is temporarily marginated along the vessel walls and is called the **marginating pool (MP)**, whereas the other half is freely circulating and is referred to as the **circulating pool (CP)**.[10] Thus, if all marginated neutrophils were to circulate freely, the total neutrophil count would double. Marginating neutrophils roll on the endothelial surface at a slow rate caused by a loose binding interaction between selectin adhesion proteins on neutrophils (L-selectin) and the L-selectin ligand on endothelial cells. The two pools are in equilibrium and rapidly and freely exchange neutrophils. Stimulants such as strenuous exercise, epinephrine, or stress can induce a shift from the MP to the CP, temporarily increasing the neutrophil count. The average neutrophil circulates ~7.5 hours in the blood before diapedesing (transendothelial migration) to the tissues, although a few die of apoptosis while in the circulation. These are occasionally seen as "necrobiotic neutrophils" with a pyknotic nucleus on the peripheral blood smear (Chapter 10).

Tissues

Most neutrophils move into the tissues from the MP in response to chemotactic stimulation (see section on Neutrophil Function). In the tissues, the neutrophil is either destroyed by trauma (cell necrosis) or lives until programmed cell death, apoptosis, occurs (Chapter 2). Neutrophils that do not receive activation signals generally die within 1–2 days. However, elevated levels of GM-CSF or G-CSF associated with an infection or inflammatory process can prolong the neutrophil life span to 3–5 days by blocking apoptosis. Senescent or apoptotic neutrophils are phagocytosed by macrophages.[9]

Neutrophil Kinetics

Neutrophils constitute the majority of circulating leukocytes. The absolute concentration varies between 1.8 and 7.0 $\times$ 10^9/L. A number of physiologic and pathologic variations affect the concentration of circulating leukocytes. Pathologic causes of changes in leukocyte numbers are discussed in subsequent chapters on white cell disorders including Chapters 21–28.

Alteration in the concentration of peripheral blood leukocytes is often the first sign of an underlying pathology. A normal leukocyte count does not rule out the presence of disease, but **leukocytosis** (an increase in leukocytes) or **leukopenia** (a decrease in leukocytes) is an important clue to disease processes and deserves further investigation including a leukocyte differential count to identify the concentration of the different

types of leukocytes. *Granulocytopenia* (granulocytes $<2.0 \times 10^9$/L) defines a decrease in all types of granulocytes (i.e., eosinophils, basophils, neutrophils). **Neutropenia** is a more specific term denoting a decrease in only neutrophils. Neutropenia in adults exists if the absolute neutrophil count falls below 1.8×10^9/L The condition of absence of granulocytes is called **agranulocytosis**, and the patient is at high risk of developing an infection. **Granulocytosis** is a term used to denote an increase in all granulocytes. **Neutrophilia** is a more specific term indicating an increase in neutrophils. Neutrophilia in adults occurs when the absolute concentration of neutrophils exceeds 7.0×10^9/L. This condition is most often the result of the body's reactive response to bacterial infection, metabolic intoxication, drug intoxication, or tissue necrosis.

Although the WBC count and absolute neutrophil count are used to evaluate neutrophil production, they reflect a transient moment in overall neutrophil kinetics and do not provide accurate, quantitative information on the rate of production or destruction, status of marrow reserves, or abnormalities in cell distribution in the tissues.

☑ CHECKPOINT 7-2

An adult patient's WBC count is 10×10^9/L and there are 90% neutrophils. What is the absolute number of neutrophils? Is this within the reference interval for neutrophils? If not, what term would be used to describe it?

Function

To be effective in its role in host defense, the neutrophil must move to the site of the foreign agent, engulf it, and destroy it. Thus, neutrophils function primarily in the tissues where microbial invasion typically occurs. Monocytes-macrophages help in this process but are slower to arrive at the site. The four steps in the innate immune response can be described as adherence, migration (chemotaxis), phagocytosis, and bacterial killing.

Adherence

Neutrophils flow freely along the vascular endothelium when neither the neutrophil nor VEC is activated. Neutrophil adherence and migration to the site of infection begin with a series of interactions between the neutrophils and VEC when these cells are activated by a variety of inflammatory mediators (cytokines).

Several different families of cell adhesion molecules (CAM) and their ligands play a major role in the adherence process. Adhesion molecules include (Table 7-3 ★):

- β2 (CD18) family of leukocyte integrins and their ligands (immunoglobulin-like CAM)
- Selectins and their ligands
- Intercellular adhesion molecules (ICAMs)

Adhesion molecules and their ligands located on the leukocytes and VEC act together to induce activation-dependent adhesion events. They are critical for every step of neutrophil recruitment to sites of tissue injury including margination along vessel walls, **diapedesis** (passage of cells through intact vessel walls), and **chemotaxis** (migration in response to a chemical stimulation). Adhesion molecules are transmembrane proteins with three domains: extracellular, transmembrane, and intracellular. A ligand's binding to the CAM extracellular domain on a neutrophil sends a signal across the membrane to the

★ **TABLE 7-3** Adhesion Molecules Important in Leukocyte–Endothelial Cell Interactions

Molecules	CD Designation	Expressed By	Counter-Receptor/Ligand
1. β_2-Integrins/neutrophils	CD11a/CD18	Activated leukocytes	ICAM-1 on VEC (CD54) ICAM-2 on VEC (CD102) ICAM-3 (CD50)
$\alpha_1\beta_2$ (LFA-1)			
$\alpha_M\beta_2$ (Mac-1)	CD11b/CD18	Activated leukocytes	ICAM-1 on EC iC3b, fibrinogen, factor X
$\alpha_x\beta_2$ (p150, 95)	CD11c/CD18	Activated leukocytes	iC3b, fibrinogen
2. Selectins			
L-selectins	CD62L	Leukocytes	Sialylated carbohydrates; PSGL-1
E-selectin	CD62E	Activated VEC	Sialylated carbohydrates (SLe^x) and L-selectin on activated leukocytes
P-selectin	CD62P	Activated VEC and platelets	PSGL-1; sialylated carbohydrates
3. Immunoglobulin supergene family			
ICAM-1	CD54	VEC	LFA-1 (CD11a-CD18)
ICAM-2	CD102	VEC	LFA-1 (CD11a-CD18)
ICAM-3	CD50	Neutrophils	VEC
PECAM-1	CD31	VEC	CD31, $\alpha_v\beta_3$
LFA-2	CD2	T lymphocytes	LFA-3 (CD58) on EC
LFA-3	CD58	VEC	CD2 on T lymphocytes
VCAM-1	CD106	VEC	VLA-4 (CD49d) on monocytes, lymphocytes, eosinophils, basophils

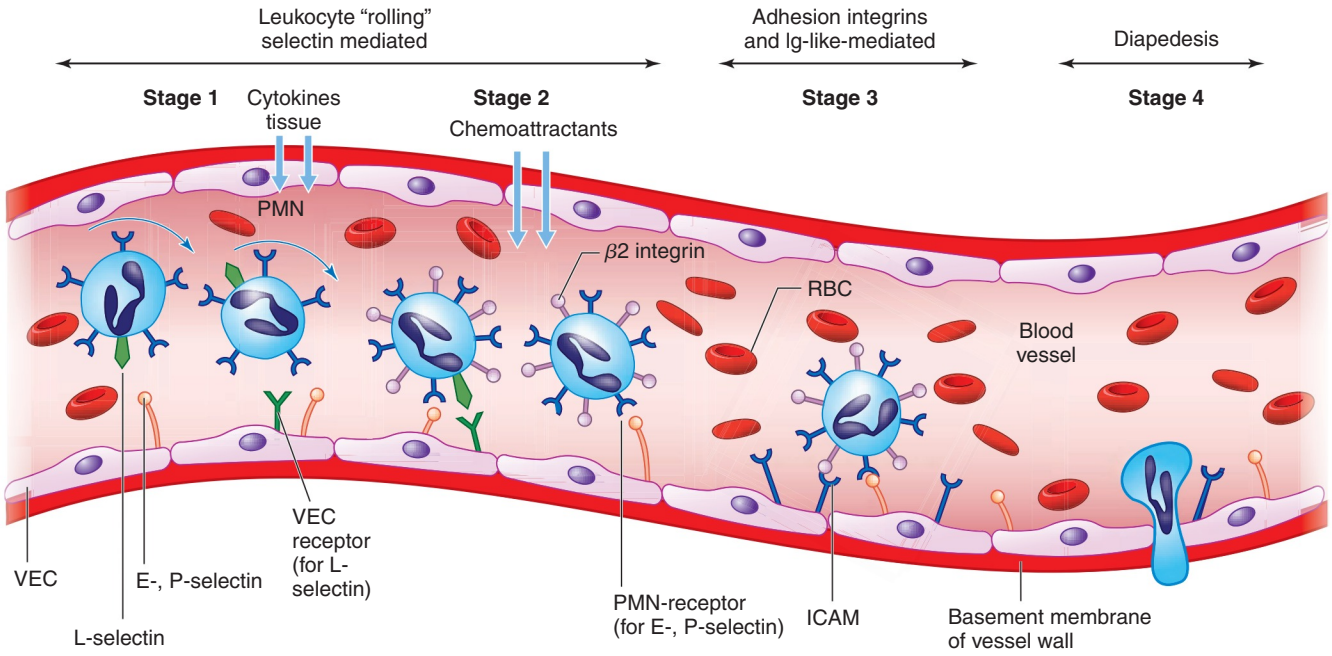
LFA = leukocyte function-related antigen; CD = cluster of differentiation; VEC = vascular endothellial cell; ICAM = intercellular adhesion moleecules; PSGL-1 = P selectin glcoprotein ligand-1

cell's interior, which activates secondary messengers within the cell. These secondary messengers affect calcium flux, NADPH oxidase activity, cytoskeleton assembly, and phagocytosis.

Neutrophils and endothelial cells are transformed from a basal state to an activated state by inflammatory mediators (cytokines and chemokines). The initial result is activation of all three classes of CAMs. Springer has proposed a model of the neutrophil-endothelial cell adhesion and migration process[12] that can be divided into four stages: (1) activation of VEC, (2) activation of neutrophils, (3) binding of neutrophils to inner vessel linings, and (4) transendothelial migration (Figure 7-6 ■).

Stage 1 involves the activation of VECs that allows for a loose association of VECs with neutrophils. Inflammatory cytokines induce the expression of E- and P-selectins and L-selectin ligands on VEC. E- and P-selectin molecules on the VEC surface interact with their ligands on the neutrophil. Additionally, L-selectin, which is constitutively present in the neutrophil membrane, interacts with its L-selectin ligands that are upregulated on the surface of the activated VEC. These interactions induce the neutrophil to transiently associate and dissociate with the VEC, causing the neutrophil to "roll" on the VEC surface. The rolling neutrophils are thus situated to respond to additional signals from chemoattractants (chemotactic substances—chemical messengers that cause directional migration of cells along a concentration gradient) generated by infectious agents or an inflammatory response.[10]

Stage 2 is the activation of neutrophils. **Chemokines** (cytokines with chemotactic activity) or other chemoattractants bind to the endothelial cell surface where they interact with the loosely bound neutrophils and result in activation of neutrophil integrins. Chemoattractants are released by tissue cells, microorganisms, and activated VEC and include specific and nonspecific proinflammatory mediators such as C5a (complement activation peptide), bacterial products, lipid mediators (e.g., platelet-activating factor [PAF]), and chemokines. Upon neutrophil activation, activation-dependent adhesion receptors including the β2-integrin molecules are expressed. Leukocyte plasma membranes have at least three β2-integrins: CD11a/CD18 (leukocyte function associated antigen-1 [LFA-1]), CD11b/CD18 (Mac-1, also known as the complement receptor 3 [CR3]), and CD11c/CD18. Each has an α subunit (CD11a, CD11b, CD11c) noncovalently linked to a β subunit (CD18).[10] The neutrophil molecule, L-selectin, is downregulated at this time.



■ **FIGURE 7-6** Adhesion of neutrophils to the vascular endothelium and eventual migration of neutrophils into the tissue occur as a result of activation of endothelial cells (EC) and neutrophils by exposure to chemoattractants. When the cells are activated, they are induced to express adhesion molecules. These transmembrane molecules send a signal across the membrane to the interior of the cell when they attach to their receptor. The process occurs in four stages: In Stage 1, E-, P-selectin and L-selectin (VEC) receptor are expressed on activated EC. The neutrophil's L-selectin and receptors for E-, P-selectin cause the neutrophil to attach loosely to the EC and roll along the endothelium. Neutrophils in Stage 2 are activated by the presence of chemoattractants in the local environment and express the β2-integrins. These chemoattractants also activate the ECs. The neutrophils in Stage 3 attach to the activated ECs via the attachment of their β2-integrins to ICAMs of the EC, resulting in a firmer attachment than in Stage 1 and halting the rolling of the neutrophil. The neutrophil in Stage 4 migrates through the endothelium and basement membrane (diapedesis) to the area of inflammation. They move in the direction of the chemoattractants (chemotaxis).

Stage 3 involves arrest of neutrophil rolling because the activated neutrophils are more tightly bound to the VECs. Expression of activation-dependent β2-integrin adhesion molecules on neutrophils mediates firm adherence to ICAMs expressed by activated VECs near the site of infection or inflammation. This induces a cytoskeletal and morphologic change in the leukocyte required for cellular migration. Stage 3 ends with a strong, sustained attachment of the leukocyte to the VEC. At this time, leukocyte NADPH oxidase membrane complexes are assembled and activated (discussed in "Bacterial Killing and/or Digestion").

Stage 4 involves the transendothelial migration phase that occurs when neutrophils move through the vessel wall at the borders of VECs by the process of diapedesis. As neutrophils pass out of the vessel and into the tissue, VECs modify their cell-to-cell adherent junctions. The neutrophils use pseudopods to squeeze between endothelial cells, leaving the vascular space and passing through the subendothelial basement membranes and periendothelial cells. Subendothelial basement membranes are presumably eroded by the secretion of the neutrophil enzymes gelatinase B and elastase from neutrophil granules. Migration is enhanced when IL-1 and/or TNF activate the VEC.[10]

An autosomal recessive disorder, leukocyte adhesion deficiency type-I (LAD-I), has partial or total absence of expression of the β2-integrins on leukocyte membranes, often due to a mutation of the CD18 gene. This results in absence of leukocyte adhesion to the VEC as well as lack of mobility and migration into the tissues and can result in life-threatening bacterial infections.[13] An inability to synthesize the E- and L-selectin ligand CD15s is seen in leukocyte adhesion deficiency type 2 (LAD-2) and also results in reoccurring infections (Chapter 21).

Migration

Once in the tissues, neutrophil migration (chemotaxis) is guided by chemoattractant gradients. Neutrophils continue their migration through the extravascular tissue, moving by directed ameboid motion toward the infected site. Locomotion of neutrophils (and other leukocytes) is a process of "crawling," not "swimming." During locomotion along a chemotactic gradient, the neutrophils acquire a characteristic asymmetric shape, a process made possible by alterations of the cytoskeleton triggered by neutrophil activation. There is an extension of a broad pseudopodium (protopod) at the anterior of the cell (containing the nucleus and organelles) and a narrow knob-like tail (uropod) at the rear of the cell. Neutrophil migration through the tissues requires β1 and β2 integrins with the continuous formation of new adhesive contacts at the cell front and detachment from the adhesive substrate at the rear of the cell. Chemotaxis is induced by a variety of chemoattractant molecules including bacterial formyl peptides (fMLP), C5a, IL-8, and PAF, many of the same molecules that activated the neutrophil during Stage 2.[12]

☑ CHECKPOINT 7-3

A patient with life-threatening recurrent infections is found to have a chromosomal mutation that results in a loss of active integrin molecules on the neutrophil surface. Why would this result in life-threatening infections?

Phagocytosis

After arriving at the site of infection, phagocytosis by neutrophils can begin. Monocytes and macrophages also arrive at the site of injury and continue to accumulate and contribute to the inflammatory process. Phagocytes must recognize the pathogen as foreign before attachment occurs and phagocytosis is initiated (Figure 7-7 ■). Once a pathogen is recognized, ingestion of the particle, fusion of the neutrophil granules with the phagosome (degranulation), and finally the process of bacterial killing and digestion occur. **Phagocytosis** is an active process that requires a large expenditure of energy by the cells. The energy required for phagocytosis can result from anaerobic glycolysis or aerobic processes.

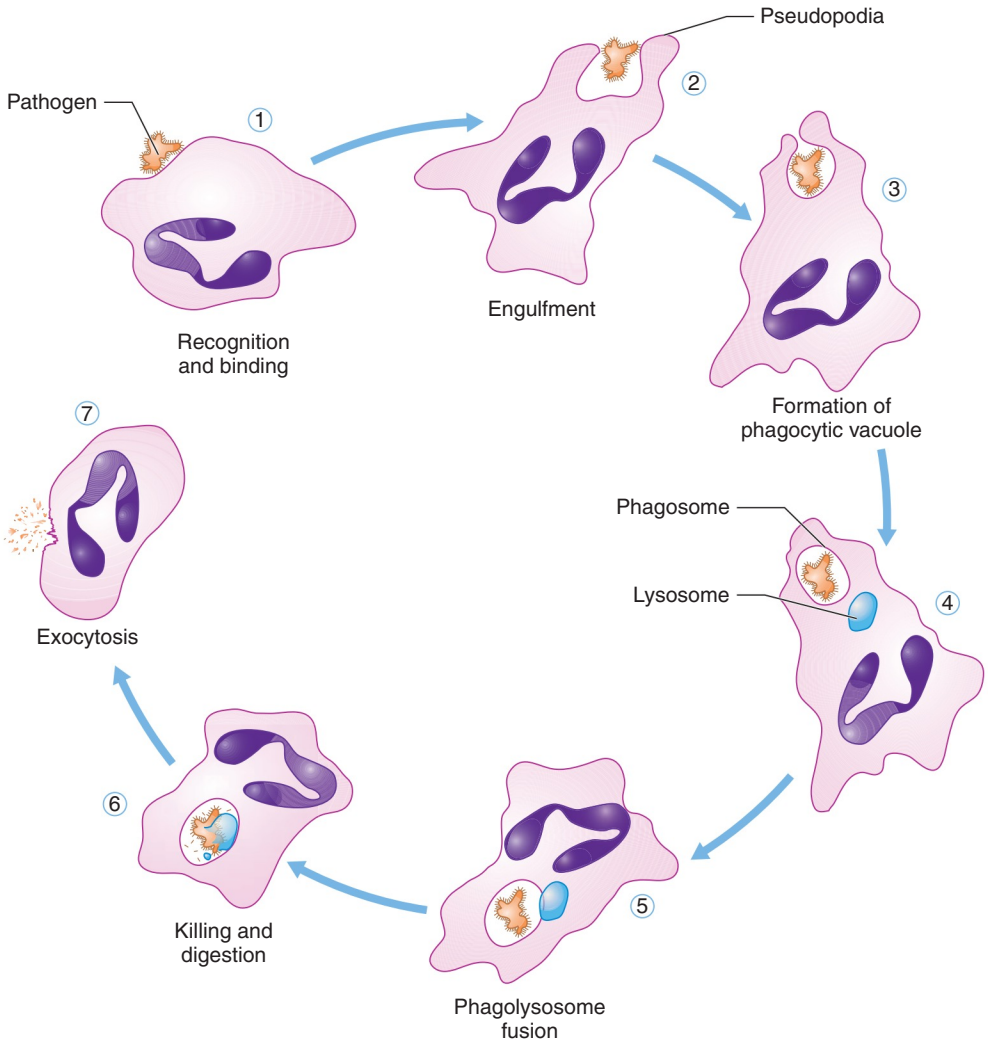
The principal factor in determining whether phagocytosis can occur is the physical nature of the surfaces of both the foreign particle and the phagocytic cell. Phagocytes recognize unique molecular characteristics of the pathogen's surface (PAMP) and bind to the invading organism via specific PRR (see the earlier section "Leukocyte Function"). Some pathogens are recognized by the process of opsonization (the coating of a particle with a soluble factor that enhances the recognition process). Enhancement of phagocytosis through the process of opsonization speeds the ingestion of particles. Two well-characterized opsonins are immunoglobulin G (IgG) and complement component C3b (see Chapter 8 for a description of IgG structure). The antibody IgG binds to the microorganism/particle by means of its Fab region, and the Fc region of the antibody attaches to three classes of Fc receptors on the neutrophil membrane (FcγRI[CD64], FcγRII[CD32], FcγRIII[CD16]). Thus, the antibody forms a connecting link between the microorganism/particle and the neutrophil. The neutrophil also has receptors for activated complement component C3b (CR1/CD35, CR3/CD11b/CD18, CR4/CD11c/CD18). Some bacteria with polysaccharide capsules avoid recognition, thus reducing the effectiveness of phagocytosis.

Following recognition and attachment, the particle is surrounded by neutrophil cytoplasmic extensions or pseudopods (Figure 7-7). As the pseudopods touch, they fuse, encompassing the particle within a phagosome that is bound by the cytoplasmic membrane turned "inside out." A plasma membrane–bound oxidase is activated during ingestion, which plays an important role in microbicidal activities.

Bacterial Killing and/or Digestion

Bacterial killing and/or digestion follows ingestion of particles. After formation of the phagosome, neutrophil granules migrate toward and fuse with the phagosome membrane, discharging their granule contents into the phagocytic vesicle (**degranulation**), forming a phagolysosome. The microbicidal and digestive proteins contained within both primary and secondary granules, which are normally sequestered from the cytoplasm of the neutrophil, are thus selectively released and activated within a membrane-enclosed system.

Microbicidal mechanisms that follow ingestion can be divided into oxygen-dependent/oxidative or oxygen-independent/nonoxidative activities (Table 7-4 ★). Oxygen-dependent microbicidal activity is most important physiologically. Phagocytosis is accompanied by an energy-dependent "respiratory burst" that generates oxidizing compounds produced from partial oxygen reduction. The respiratory burst is described as including a significant increase in glycolysis, a 2–3-fold increase in oxygen consumption, a 2–10-fold



■ **FIGURE 7-7** Phagocytosis begins with (1) recognition and attachment of the pathogen to the neutrophil (or macrophage). The pathogen is then internalized (2), forming a phagocytic vacuole (phagosome [3]). Next, a primary granule (lysosome) fuses with the vacuole (4), forming a phagolysosome (5). The granule releases its contents (6) into the vacuole to help kill and digest the microbe (degranulation). This is followed by extrusion of undigested vacuole contents from the neutrophil (exocytosis [7]).

★ **TABLE 7-4** Neutrophil Antimicrobial Systems

Oxygen Dependent	Oxygen Independent
• Myeloperoxidase independent Hydrogen peroxide (H_2O_2) Superoxide anion (O_2^-) Hydroxyl radicals (OH^-) Singlet oxygen ($^1O_2$) • Myeloperoxidase dependent (forms oxidized halogens)	• Acid pH of phagosome: antibacterial; enhances some oxygen-dependent antimicrobial mechanisms and other enzymes • Lysozyme (primary and secondary granules): hydrolyzes cell wall of some bacteria; digests killed microbes • Lactoferrin (secondary granules): binds iron necessary for bacterial growth; also directly bactericidal • BPI (bactericidal permeability-inducing protein; primary granules); Coat microbes; alters cell permeability • Defensins: small cationic peptides; broad spectrum of bactericidal activity • Collagenase (secondary granules): degrades microbe macromolecules • Hydrolases (primary granules): digests microbe

increase in hexose monophosphate (HMP) shunt activity and generation of NADPH, and the production of a series of reactive oxygen species (ROS).[14] The oxidizing compounds—ROS—are important agents in killing ingested organisms. The enzyme activity needed to generate ROS is provided by NADPH oxidase, also known as *respiratory burst oxidase*. In resting cells, NADPH oxidase is found as separate components of the plasma membrane (gp^{91phox}, p^{22phox}, Rap^{1}) and intracellular stores (p^{47phox}, p^{67phox}).[15] When phagocytosis takes place, the plasma membrane is internalized so that what was originally the outer plasma membrane surface is now the lining of the phagocytic vesicle and faces the interior of the phagosome. When the resting cell is exposed to any of a wide variety of activating stimuli, activated NADPH oxidase is assembled from the cytoplasmic and membrane-associated subunits at the phagosome membrane. From this location, the NADPH oxidase generates and pours ROS into the phagosome.

Once assembled, the oxidase produces superoxide anion (O_2^-) and $NADP^+$. The $NADP^+$ activates the HMP shunt (Chapter 5), generating more NADPH. O_2^- is further metabolized to produce additional ROS with increasing microbicidal potency.

$$2\ NADPH + 2\ O_2 \xrightarrow{\text{NADPH oxidase}} 2\ NADP^+ + 2\ O_2^- + 2\ H^+$$

$$2\ H^+ + O_2^- + O_2^- \xrightarrow{\text{Superoxide oxidase}} H_2O_2 + O_2$$

$$H_2O_2 + O_2^- \rightarrow 2\ OH^- + {}^1O_2$$

The activated oxidase can be detected in the laboratory by a nitroblue tetrazolium (NBT) test, cytochrome reduction, or chemiluminescence test.

The second oxygen-dependent microbicidal system involves the neutrophil's primary granule enzyme myeloperoxidase (MPO). Myeloperoxidase catalyzes the interaction of hydrogen peroxide produced during the respiratory burst with halide ions (e.g., chloride/Cl^-) giving rise to oxidized halogens (e.g., hypochlorous acid/HOCl) that increase bacterial killing.[14]

$$H_2O_2 + Cl^- \xrightarrow{MPO} OCl^- + H_2O$$

The oxidants generated by the respiratory burst have potent microbicidal activity against a wide variety of microorganisms such as bacteria, fungi, and multicellular and unicellular parasites. However, the phagocyte and surrounding tissues are also susceptible to damage. To detoxify the oxidant radicals, phagocytes use a variety of mechanisms such as superoxide dismutase, catalase, and a variety of other antioxidants.

Oxygen-independent granule proteins present in primary, secondary, and tertiary granules of neutrophils can successfully kill and degrade many strains of both gram-negative and gram-positive bacteria. See Table 7-4 for a list of the most important nonoxygen-dependent antimicrobial proteins of neutrophils. Initially, the pH within the phagolysosome decreases and inhibits bacterial growth, but this alone is insufficient to kill most microbes. Acidic conditions, however, can enhance the activity of some granule proteins such as hydrolases and lactoferrin, which perform optimally at low pH. In the extracellular environment, microorganisms that escape phagocytosis are also subject to killing by reactive oxygen metabolites such as H_2O_2 that form from the O_2^- secreted by active plasma membrane NADPH complexes into the tissue matrices. Neutrophils are not resistant to the toxic effects of the oxidants they secrete and thus have high mortality during any sustained inflammatory response.[16]

In patients with chronic granulomatous disease, neutrophils are missing one of the components of the NADPH oxidase complex and therefore fail to produce the respiratory burst. They are still capable of eliminating infection caused by strains of bacteria susceptible to killing by oxygen-independent mechanisms, but this antimicrobial system is not very effective alone. Often these patients eventually die of multiple infections with bacteria resistant to the killing actions of these granule proteins (Chapter 21).

In addition to their primary functions of phagocytosis and killing of microorganisms, neutrophils interact in other physiologic processes. Neutrophils stimulate coagulation by releasing a substance that activates prekallikrein to kallikrein, which in turn cleaves kinins from high-molecular-weight kininogen. Kinins are responsible for vascular dilation and increased vessel permeability. Kinins are also chemotactic molecules that attract neutrophils to sites of inflammation. Neutrophils initially activate kinin production, but as the cells accumulate, they break down kinins. Neutrophils also secrete interleukin-1 (IL-1), a pyrogen that acts on the hypothalamus to produce fever.

☑ CHECKPOINT 7-4

A patient has a compromised ability to utilize the oxygen-dependent pathway in neutrophils. What two important microbial killing mechanisms could be affected?

EOSINOPHILS

The eosinophil originates from the IL-5–responsive $CD34^+$ myeloid progenitor cells (CFU-Eo). Cytokines that influence proliferation and differentiation of the eosinophil lineage include GM-CSF, IL-3, and IL-5.[17] However, it is now recognized that IL-5, released largely by activated T_H2 lymphocytes and in small amounts by eosinophils, mast cells, NK cells, and natural killer T (NKT) cells, has relative lineage specificity for eosinophils and is the major cytokine required for eosinophil production and terminal differentiation.[18]

Differentiation, Maturation, and Morphology

The eosinophil undergoes a morphologic maturation similar to the neutrophil with the same six stages of maturation identified. However, it is not possible to morphologically differentiate eosinophilic precursors from neutrophilic precursors with the light microscope until the myelocyte stage. At this stage, the typical acidophilic crystalloid granules of the eosinophil appear. Granule formation begins in the promyelocyte with small primary granules that lack the crystalloid core of the specific granules. The first two stages (eosinophilic myeloblast and promyelocyte) will not be described because they are morphologically identical to the neutrophilic myeloblast and promyelocyte.

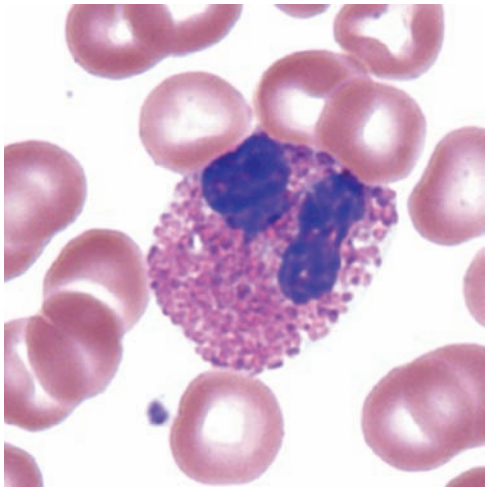
Eosinophilic Myelocyte to Mature Eosinophil

The eosinophilic myelocyte contains large, eosin-staining, crystalloid granules. Maturation from the myelocyte to the metamyelocyte, band, and segmented eosinophil stage is similar to that described for the neutrophils with gradual nuclear indentation and segmentation. No appreciable change occurs in the cytoplasm in these later stages of development. The reddish orange spherical granules are larger than neutrophilic granules, uniform in size, and evenly distributed throughout the cell. Because of the low percentage of eosinophils in the bone marrow, differentiating the eosinophil into its maturational stages (e.g., eosinophilic myelocyte) serves no useful purpose when the count is normal. Bone marrow maturation and storage time are about 9 days.

The mature eosinophil (Figure 7-8 ■) is 12–15 mcM in diameter. The nucleus usually has no more than two or three lobes, and the cytoplasm is completely filled with granules. The mature eosinophil contains three types of granules: primary granules, small granules, and specific or secondary granules.

Primary granules contain **Charcot–Leyden crystal** proteins (also called *galectin-10*) that possess lysophospholipase activity. The Charcot–Leyden crystal proteins are found in a variety of tissues, body fluids, and secretions in association with eosinophilic inflammatory reactions.[19] Small granules contain the enzymes acid phosphatase and arylsulphatase but are not well characterized.

Specific granules are the primary source of the cytotoxic and proinflammatory properties of the eosinophil.[18] Specific granules are large, bound by a phospholipid membrane, and have a central crystalloid core surrounded by a matrix. These granules contain four major proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (Table 7-5 ★). The MBP is located in the crystalloid core; the other three proteins are found in the granule matrix. The crystalloid core also appears to store a number of proinflammatory cytokines such as IL-2, IL-4, and GM-CSF, and the matrix contains IL-5 and TNF-α. The eosinophil has the capacity to synthesize and elaborate a number of other cytokines as well. The eosinophil's capacity to produce cytokines has led to increased interest and research into the eosinophil's role as an effector cell in allergic inflammation. In addition to granules, the eosinophil, like the neutrophil, contains a



■ **FIGURE 7-8** Eosinophil (peripheral blood, Wright-Giemsa stain, 1000× magnification).

★ **TABLE 7-5** Major Constituents of Eosinophil Granules

Protein	Characteristics
Major basic protein (MBP)	Is cytotoxic for protozoans and helminth parasites Stimulates release of histamine from mast cells and basophils Neutralizes mast cell and basophil heparin
Eosinophil cationic protein (ECP)	Is capable of killing mammalian and nonmammalian cells Stimulates release of histamine from mast cells and basophils Inhibits T lymphocyte proliferation Activates plasminogen Enhances mucus production in the bronchi Stimulates glycosaminoglycan production by fibroblasts
Eosinophil-derived neurotoxin (EDN)	Can provoke cerebral and cerebellar dysfunction in animals Inhibits T-cell responses
Eosinophil peroxidase (EPO)	Combines with H_2O_2 and halide ions to produce a potent bactericidal and helminthicidal action Is cytotoxic for tumor and host cells Stimulates histamine release and degranulation of mast cells Diminishes roles of other inflammatory cells by inactivating leukotrienes
Lysophospholipase	Forms Charcot-Leyden crystals
Miscellaneous enzymes	Phospholipase D: inactivates mast cell PAF Arylsulphatase: inactivates mast cell (leukotriene D4) Histaminase: neutralizes mast cell histamine Acid phosphatase, catalase, nonspecific esterases
Lipid-derived mediators	Promote smooth muscle contraction and mucus secretion and inhibit mast cell degranulation PAF; thromboxane B2

number of lipid bodies that increase during eosinophil activation in vitro.[17,18] Eosinophils express CD9, CD-11a, -11b, -11c, and CD13 molecules on their cytoplasmic membrane. These molecules function in antigen presentation, VEC adhesion, and transmigration into the tissues, respectively. Additionally, the primary receptors that impart the unique functional features of eosinophils are interleukin-5 receptor subunit-α ([IL-5Rα] responsible for proliferation, activation, and survival), CC-chemokine receptor 3 ([CCR3] promoting chemotaxis in response to eotaxins), and sialic acid-binding immunoglobulin-like lectin 8 ([SIGLEC8] whose signaling induces apoptosis).[18,20]

Distribution, Concentration, and Kinetics

Eosinophils in adults have a concentration in the peripheral blood $\leq 0.40 \times 10^9$/L. The cell shows a diurnal variation with highest concentration in the morning and the lowest concentration in the evening.[21] Eosinophilia in adults is defined as $>0.40 \times 10^9$/L and is associated with allergic diseases, parasitic infections, toxic reactions, gastrointestinal diseases, respiratory tract disorders, neoplastic disorders, and other conditions. (See Chapter 21 for a complete list.) Eosinophilia is T-cell dependent because T cells are the predominant source of IL-5. Persistent eosinophilia is seen in hypereosinophilic syndrome, a myeloproliferative disorder (Chapter 24).

Very little is known about the kinetics of eosinophils. Most of the body's eosinophil population lies in connective tissue below the epithelial layer in tissues that are exposed to the external environment such as the nasal passages, lung, skin, gastrointestinal tract,[22] and urinary tract. These cells spend ~18 hours in the peripheral blood before migrating to the tissues where they can live for several weeks. Once in the tissues, eosinophils do not re-enter the circulation.[18]

Function

The cellular arm of the adaptive immune system (T lymphocytes; Chapter 8) influences eosinophil production and function.[23,24] Eosinophils are pro-inflammatory cells associated with allergic diseases, parasitic infections, and chronic inflammation. Their major role is host defense against helminth parasites via a complex interaction of eosinophils, the adaptive immune system, and parasite. The eosinophil adheres to the organism and releases its granule contents onto the surface of the parasite via exocytosis. A number of eosinophil proteins, including MBP, ECP, and EPO, are highly toxic for larval parasites. Eosinophils are also capable of phagocytizing bacteria (although less efficiently than neutrophils and macrophages) and have been shown to function as antigen-presenting cells.[25]

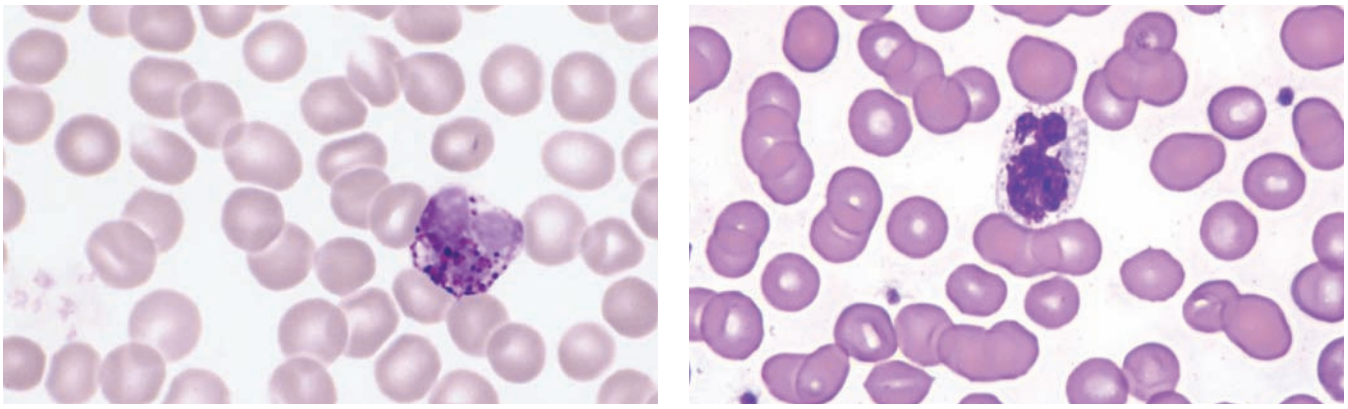
Eosinophils respond weakly to IL-3, IL-5, and GM-CSF as chemotaxins, but IL-5 synthesized by T lymphocytes has been shown to strongly prime eosinophils for a chemotactic response to PAF, leukotriene B_4, or IL-8. Products released from basophils and mast cells (eosinophil chemotactic factor [ECF]), lymphokines from sensitized lymphocytes, and allergy-related antigen–antibody complexes are strongly chemotactic for eosinophils. Eosinophils express Fc receptors for IgE, the immunoglobulin that is prevalent in the response to parasitic infections and mediates activation of eosinophil killing mechanisms. The cytokines IL-3, IL-5, and GM-CSF promote the adherence of eosinophils to VEC; transendothelial migration is 10 times higher in the presence of these cytokines.

Eosinophils have a $\beta 2$ integrin-independent mechanism for recruitment into the tissues that appears to be modulated by the eosinophil adhesion receptor, VLA-4, and its ligand VCAM-1, found on VECs that have been activated by IL-1, TNF, or IL-4. Changes in eosinophil adhesion molecule expression occur during eosinophil migration. This implies that dynamic changes in cell adhesion molecules are involved in cell recruitment to areas of inflammation.

The eosinophil liberates substances that can neutralize mast cell and basophil products, thereby down modulating the allergic response (Table 7-5). Increasing evidence suggests a direct correlation between the degree of eosinophilia and severity of inflammatory diseases, such as asthma, in which eosinophil activation and degranulation contribute to the characteristic features of mucous production, bronchoconstriction, and tissue remodeling.[18] In inflammatory conditions, the cytotoxic potential of eosinophils is turned against the host's own tissue.[24]

BASOPHILS

Basophils (Figure 7-9 ■) originate from the $CD34^+$ myeloid progenitors in the bone marrow. IL-3 is the main cytokine involved in human basophil growth and differentiation, but GM-CSF, stem cell factor (SCF), IL-4, and IL-5 can also be involved.[26,27]



a b

■ **FIGURE 7-9** **(a)** Basophil (peripheral blood, Wright-Giemsa stain, 1000× magnification). **(b)** Basophil with washed out granules (Wright-Giemsa stain, staining artifact).

Differentiation, Maturation, and Morphology

Basophils undergo a maturation process similar to that described for the neutrophil. The first recognizable stage is the promyelocyte, although this stage is very difficult to differentiate from the promyelocyte of the neutrophil or eosinophil. As with eosinophils and neutrophils, the various stages of the maturing basophil are characterized by a gradual indentation and segmentation of the nucleus.

Basophilic Myelocyte to Mature Basophil

The basophilic myelocyte, metamyelocyte, band, and segmented form are easily differentiated from other granulocytes by the presence of the large purple-black granules unevenly distributed throughout the cytoplasm. The granules are described as metachromatic and contain histamine, heparin, cathepsin G, major basic protein, and lysophospholipase.[26] The mature basophil ranges in size from 10–15 mcM and has a segmented nucleus and many purple granules obscuring both the background of the cytoplasm and the nucleus. Basophil granules contain peroxidase and are positive with the PAS cytochemical reaction. Basophil granules are water-soluble and can dissolve on a well-rinsed Wright-stained smear, resulting in clear areas within the cytoplasm. Usually a few deep-purple–staining granules remain to aid in the identification of the cell. Basophils express CD9, CD11a, and CD13 molecules on their cytoplasmic membrane.

Mast Cell

The relationship between basophils and mast cells continues to be investigated. Research shows that basophils and mast cells represent distinct, terminally differentiated cells, separately derived from the $CD34^+$ common myeloid progenitor cell. Distinct committed progenitor cells (CFU-Ba and CFU-MC) have been identified for each lineage.[28] Mast cells are found in the bone marrow and tissues but are not found in peripheral blood. Mast cells have proliferative potential and live for several weeks to months. At times, differentiating the mast cell and the basophil precursors in the bone marrow is difficult although some differences exist (Table 7-6 ★). The mast cell nucleus is round and surrounded by a dense population of granules. The mast cell granules contain acid phosphatase, protease, and alkaline phosphatase. Mast cells have a membrane antigen profile similar to that of macrophages.

Concentration, Distribution, and Destruction

Basophils' maturation in the bone marrow requires 2.5–7 days before they are released into circulation. In the peripheral blood, they number $<0.2 \times 10^9$/L (<1% of the total leukocytes). Basophilia in adults is defined as $>0.2 \times 10^9$/L in the peripheral blood. Basophils are end-stage cells incapable of proliferation and spend only hours in the peripheral blood.

Function

Both basophils and mast cells function as mediators of inflammatory responses, especially those of immediate hypersensitivity reactions such as asthma, urticaria, allergic rhinitis, and anaphylaxis. These cells have membrane receptors for IgE (FcεR). When IgE attaches to the receptor, the cell is activated and degranulation is initiated. Degranulation releases enzymes that are vasoactive, bronchoconstrictive, and chemotactic (especially for eosinophils). This release of mediators initiates the classic clinical signs of immediate hypersensitivity reactions. These cells can synthesize more granules after degranulation occurs. Basophils and mast cells express CD40 ligand (CD40L) that interacts with CD40 molecules expressed on B lymphocytes. In conjunction with IL-4, the interaction of B lymphocyte CD40 and basophil CD40L can induce IgE synthesis by B lymphocytes. Thus, basophils can play an important role in inducing and maintaining allergic reactions.[27]

☑ CHECKPOINT 7-5

Indicate which of the granulocytes will be increased in the following conditions: a bacterial infection, an immediate hypersensitivity reaction, and an asthmatic reaction.

MONOCYTES

The monocyte is produced in the bone marrow from a bipotential progenitor cell, the GMP, which is capable of producing either mature monocytes or neutrophils. The differentiation and proliferation of GMP into monocytes depend on the action of GM-CSF, IL-3, and M-CSF. The primary role of monocytes is host defense and this

★ **TABLE 7-6** Comparison of the Characteristics of Basophils and Mast Cells

Characteristics	Basophils	Mast Cells
Origin	Hematopoietic stem cell	Hematopoietic stem cell
Site of maturation	Bone marrow	Connective or mucosal tissue
Proliferative potential	No	Yes
Life span	Days	Weeks to months
Size	Small	Large
Nucleus	Segmented	Round
Granules	Few, small (peroxidase positive)	Many, large (acid phosphatase, alkaline phosphatase positive)
Key cytokine regulating development	IL-3	SCF
Surface receptors:		
IL-3-R	Present	Absent
c-kit (SCF-R)	Absent	Present
IgE receptor (FcεR)	Present	Present

role is fulfilled in the tissues. Monocytes continue to differentiate in the tissues, transforming into macrophages. Monocytes and macrophages can be stimulated by T lymphocytes and endotoxin to liberate endogenous M-CSF, which can be one mechanism for the monocytosis associated with some infections. M-CSF also activates the secretory and phagocytic activity of monocytes and macrophages.[29] Monocytes and macrophages make up the **monocyte-macrophage system**, also called the **mononuclear phagocyte (MNP) system**.

Differentiation, Maturation, and Morphology

The morphologically recognizable monocyte precursors in the bone marrow are the monoblast and the promonocyte. These cells are present in a very low concentration in normal bone marrow and are found in abundance only in leukemic processes involving the MNP system. The monoblast of the marrow cannot be morphologically distinguished from the myeloblast by light microscopy unless proliferation of the monocytic series is marked as occurs in monocytic leukemia. Because myeloblasts and monoblasts are indistinguishable by light microscopy, cytochemical stains (Chapters 23, 26, 37), and immunophenotyping (Chapters 23, 26, 40) frequently are used to help differentiate myeloblasts and monoblasts in suspected cases of leukemia.

Monoblast

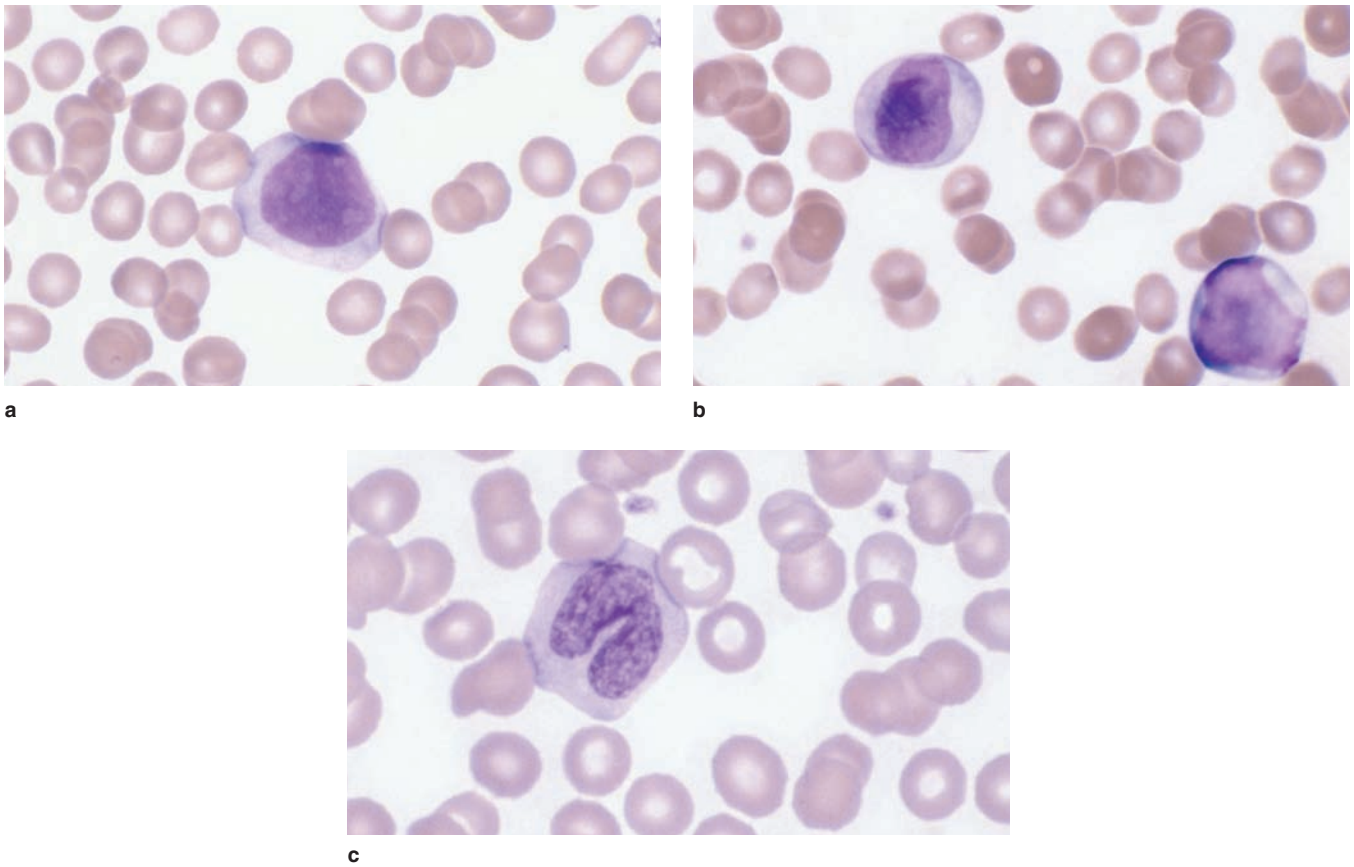
The monoblast (Figure 7-10a ■) nucleus is most often ovoid or round but can be folded or indented. Monoblasts are large (12–20 mcM in diameter). The pale blue-purple nuclear chromatin is finely dispersed (lacy), and several nucleoli are easily identified. The monoblast has abundant agranular blue-gray cytoplasm.

Promonocyte

The promonocyte (Figure 7-10b ■) is an intermediate form between the monoblast and the monocyte. The promonocyte is usually the first stage to develop morphologic characteristics that allow it to be clearly differentiated as a monocyte precursor by light microscopy. The cell is large, 12–20 mcM in diameter. The nucleus is most often irregular and indented with a fine chromatin network. Nuclear chromatin is coarser than the monoblast, and nucleoli can be present. The promonocyte's cytoplasm is abundant with a blue-gray color; azurophilic granules can be present. Cytochemical stains for nonspecific esterase, peroxidase, acid phosphatase, and arylsulfatase are positive.

Monocyte

Mature monocytes (Figure 7-10c ■) range in size from 12–20 mcM with an average size of 18 mcM, making them the largest mature cells in peripheral blood. The nucleus is frequently horseshoe- or



a **b** **c**

■ **FIGURE 7-10** Stages of monocyte maturation: **(a)** monoblast: Note lacy chromatin, nucleoli, and high N:C ratio; **(b)** promonocyte: The chromatin is somewhat more coarse and the amount of cytoplasm is increased; **(c)** monocyte: The nucleus is more lacy than that of a neutrophil or lymphocyte and is irregular in shape ([a, b]: bone marrow, Wright-Giemsa stain, 1000× magnification. [c]: peripheral blood, Wright-Giemsa stain, 1000× magnification).

bean-shaped and possesses numerous folds, giving it the appearance of brainlike convolutions or chewed gum. The chromatin is loose and linear, forming a lacy pattern in comparison to the clumped dense chromatin of mature lymphocytes or granulocytes. Monocytes, however, are sometimes difficult to distinguish from large lymphocytes, especially in reactive states when there are many reactive lymphocytes. The monocyte cytoplasm has variable morphologic characteristics depending on its activity. The cell adheres to glass and "spreads" or sends out numerous pseudopods, resulting in a wide variation of size and shape on blood smears. The blue-gray cytoplasm is evenly dispersed with fine, dustlike membrane-bound granules, which give the cell cytoplasm the appearance of ground glass. Vacuoles are frequently observed in the cytoplasm.

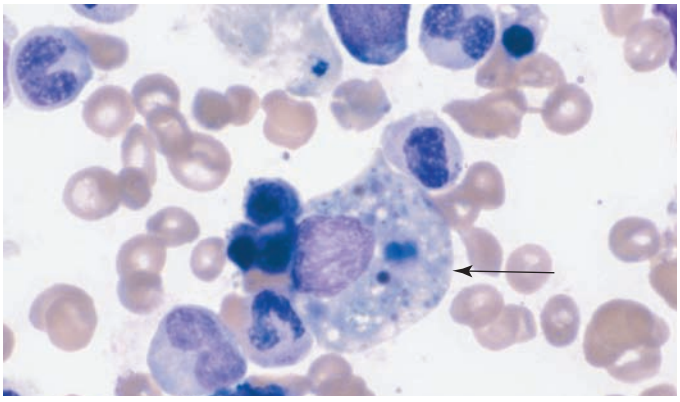
Electron-microscopic cytochemistry reveals two types of granules present in monocytes. One type contains peroxidase, acid phosphatase, and arylsulfatase, suggesting that these granules are similar to the lysosomes (primary azurophilic granules) of neutrophils. Less is known about the content of the other type of granule except that they do not contain alkaline phosphatase and are therefore dissimilar to specific granules of neutrophils.[30] The lipid membrane of the granules stains faintly with Sudan black B. Many CD markers including CD11b/CD18, CD13, CD14, and CD15 are expressed by monocytes.

Macrophage

The monocyte leaves the blood and enters the tissues where it matures into a macrophage (Figure 7-11 ■). The transition from monocyte to macrophage is characterized by progressive cellular enlargement, reaching a size of 15–80 mcM. The nucleus becomes round with a reticular (netlike) appearance, nucleoli appear, and the cytoplasm appears blue-gray with irregular edges and many vacuoles present. As it matures, the macrophage loses peroxidase, but the amount of endoplasmic reticulum (ER), lysosomes, and mitochondria all increase. In addition, distinct granules are noted in the maturing macrophage and are found to contain lysosomal hydrolases. Macrophages acquire the expression of CD68, a glycoprotein that can be important in lipid metabolism. These cells can live for months in the tissues. Macrophages do not normally re-enter the blood, but in areas of inflammation, some can gain access to the lymph, eventually entering the circulation.

Tissue macrophages, also known as *histiocytes*, develop different cytochemical and morphologic characteristics that depend on the site of maturation and habitation in tissue. These cells are widely distributed in the body and have been given specific names depending on their anatomic location. For example, macrophages in the liver are known as *Kupffer cells*, those in the lung as *alveolar macrophages*, those in the skin as *Langerhans cells*, and those in the brain as *microglial cells*. The osteoclasts in the bone are also of MNP derivation.[31]

Macrophages can proliferate in the tissues, especially in areas of inflammation, thereby increasing the number of cells at these sites. Occasionally, two or more macrophages fuse to produce giant multinucleated cells. This occurs in chronic inflammatory states and granulomatous lesions where many macrophages are tightly packed together. Fusion also occurs when particulate matter is too large for one cell to ingest or when two cells simultaneously ingest a particle.



■ **FIGURE 7-11** Arrow indicates a macrophage. Note the numerous vacuoles and cellular debris (bone marrow, Wright-Giemsa stain, 1000× magnification).

Distribution, Concentration, and Kinetics

Before maturing into monocytes, the promonocyte undergoes two or three divisions. Bone marrow transit time is ~54 hours. In contrast to the large neutrophil storage pool, there is no significant reserve pool of monocytes in the bone marrow. Most monocytes are released within a day after their maturation from promonocytes. Monocytes diapedese into the tissue from the peripheral blood in a random manner after circulating for an average transit time of ~8 hours.[29]

Similar to neutrophils, the total vascular monocyte pool consists of a marginated pool and a circulating pool. However, unlike neutrophils, the marginating pool is about three times the size of the circulating pool. Monocytes in the circulating peripheral blood number about $0.1–0.8 \times 10^9$/L in the normal adult, or ~2–10% of the total leukocytes. Children have a slightly higher concentration. Monocytosis (increase in monocytes) in adults occurs when the absolute monocyte count is $>0.8 \times 10^9$/L.

Function

Monocytes and macrophages are active in both the innate and adaptive IR. In addition to their phagocytic function, they secrete a variety of substances that affect the function of other cells, especially lymphocytes. Lymphocytes in turn secrete soluble products (lymphokines) that modulate monocytic functions.

Monocytes and macrophages ingest and kill microorganisms. They are particularly effective in inhibiting the growth of intracellular microorganisms, a process that first requires monocyte activation. Activation results in the production of many large granules, enhanced phagocytosis, and an increase in the activity of the HMP shunt. Monocyte activation occurs in the presence of lymphokines produced by T lymphocytes. Killing by activated monocytes is nonspecific (i.e., the secretions from *Listeria*-sensitized T cells activate a killing mechanism in monocytes not only to *Listeria* but also to other

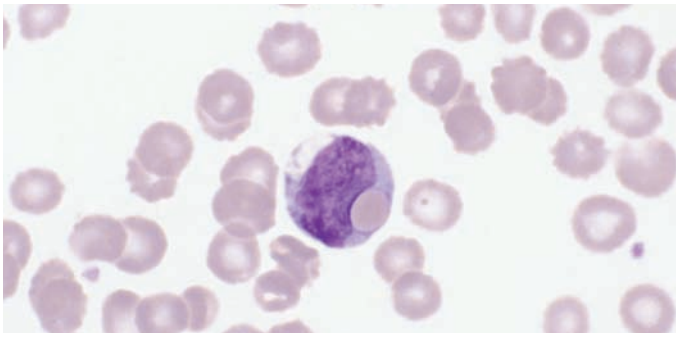
microorganisms). Activation can also occur as the result of the actions of other substances on monocytes such as endotoxins and naturally occurring opsonins.

Monocytes/macrophages have some ability to bind directly to microorganisms via PAMP and PRR (see the section "Leukocyte Function"), but binding is enhanced if the microorganism has been opsonized by complement or immunoglobulin (Web Figure 7-1). Macrophages possess receptors for the Fc component of IgG and for the complement component C3b. Following attachment to an opsonized organism, ingestion occurs in a manner similar to that of neutrophils (Figure 7-7). Primary lysosomes fuse with the phagosome, releasing hydrolytic enzymes and other microbicidal substances, the most powerful of which are products of oxygen metabolism—superoxide anion (O_2^-), hydroxy radical (OH^-), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2)—generated in a reaction analogous to the neutrophil respiratory burst.

In addition to microbicidal activity, activated macrophages also attach to tumor cells and kill them by a direct cytotoxic effect. If the tumor cell has immunoglobulin attached, the macrophage Fc receptor attaches to the Fc portion of the immunoglobulin and exerts a lytic effect on the tumor cell.

Macrophages are important scavengers, phagocytizing cellular debris, aging cells, and other particulate matter. Monocytes in the blood ingest activated clotting factors, thus limiting the coagulation process. They also ingest denatured protein and antigen–antibody complexes. Macrophages lining the blood vessels remove toxic substances from the blood, preventing their escape into tissues. The macrophages of the spleen are important in removing aged erythrocytes from the blood; they conserve the iron of hemoglobin by either storing it for future use or releasing it to transferrin for use by developing erythroblasts in the marrow (Chapter 6). By virtue of their Fc receptor, the splenic macrophages also remove cells sensitized with antibody. In autoimmune hemolytic anemias or in autoimmune thrombocytopenia, the spleen is sometimes removed to prevent premature destruction of these antibody-coated cells in an attempt to alleviate the resulting cytopenias.

For a variety of reasons, sometimes unknown, erythrocytes in some pathologic conditions are randomly phagocytosed and destroyed by monocytes and macrophages in the blood and bone marrow (erythrophagocytosis) (Figure 7-12 ■).



■ **FIGURE 7-12** Erythrophagocytosis by a monocyte (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Erythrophagocytosis is readily identified when the ingested erythrocytes still contain hemoglobin. At times, erythrocyte digestion can be inferred by finding ghost spheres within the macrophage.

In addition to its role in pathogen control and tissue homeostasis, the MNP plays a major role in initiating and regulating the adaptive IR.[29] Macrophages phagocytize and degrade both soluble and particulate substances that are foreign to the host. They process the degraded substances, generating fragments containing *antigenic determinants* or *epitopes* that are bound by MHC molecules on the macrophage membrane and presented to T lymphocytes. Thus, monocytes and macrophages can function as antigen-presenting cells (APCs) in the adaptive immune system. In addition to antigen presentation, the macrophage produces a number of cytokines that regulate the adaptive IR as well as the inflammatory response. Antigen-specific T lymphocyte proliferation requires antigen presentation in context with cell surface MHC antigens and stimulation with soluble mediators such as IL-1 and IL-2. T lymphocytes respond to foreign antigens only when the antigens are displayed on APCs that have the same MHC phenotype as the lymphocyte.

Macrophages stimulate the proliferation and differentiation of lymphocytes through secretion of cytokines. They secrete IL-1, which stimulates T lymphocytes to secrete interleukin-2 (IL-2), a growth factor that stimulates the proliferation of other T lymphocytes. In addition, IL-2 acts in synergy with interferon (IFN) to activate macrophages. When released from macrophages, arachidonic metabolites (e.g., leukotrienes, prostaglandins) inhibit the function of activated lymphocytes. Activated lymphocytes in turn secrete lymphokines that regulate the function of macrophages. For these interdependent reactions to occur between the macrophage and lymphocyte, the two cell populations must express compatible MHC antigens.

In addition to IL-1, macrophages release a variety of substances that are involved in host defense or that can affect the function of other cells. Other secretory products involved in host defense include lysozyme, complement components, and IFN (an antiviral compound). Secreted substances that modulate other cells include hematopoietic growth factors (e.g., G-CSF, M-CSF, GM-CSF), substances that stimulate the growth of new capillaries (angiogenic cytokines), factors that stimulate and suppress the activity of lymphocytes, chemotactic substances for neutrophils, and a substance that stimulates the hepatocyte to secrete fibrinogen. After death, activated macrophages also release enzymes such as collagenase, elastase, and neutral proteinase that hydrolyze tissue components.

☑ CHECKPOINT 7-6

An adult patient's neutrophil count and monocyte count are extremely low ($<0.5 \times 10^9$/L and $<0.05 \times 10^9$/L, respectively). What body defense mechanism is at risk?

Summary

Leukocytes include five morphologically and functionally distinct types of nucleated blood cells: neutrophils, eosinophils, basophils, monocytes, and lymphocytes, all of which develop from the pluripotential hematopoietic stem cells in the bone marrow. Under the influence of hematopoietic growth factors, the HSC matures into terminally differentiated cells. These cells leave the bone marrow and enter the circulation. The two pools of neutrophils in the blood are circulating and marginated pools. About one-half of the neutrophils are in each pool. Monocytes also exist in two pools, but the marginating pool is about three times the size of the circulating pool. Leukocytes generally circulate only a matter of hours in the peripheral blood before diapedesing into the tissues. Normally, only mature forms are found in the peripheral blood but immature forms can be seen in newborns and in a variety of diseases. The adult reference interval for total WBC count is 4.5–11.0 $\times$ 10^9/L. Newborns have higher counts than adults (9–30 $\times$ 10^9/L). In the circulation, neutrophils are the most numerous cells followed by lymphocytes, monocytes, eosinophils, and basophils. An increase or decrease in leukocytes can be the result of an increase or decrease in all cell types or, more commonly, in just one cell type. When the WBC count is abnormal, a differential should be performed to determine which cell type is increased or decreased. Both the absolute and relative (%) cell type concentrations should be determined and reported.

Leukocytes serve as the defenders of the body against foreign invaders and noninfectious challenges by participating in phagocytosis (innate IR) and the adaptive IR. Leukocytes are attracted to sites of inflammation, infection, or tissue injury by chemoattractants and leave the circulation using special adhesion molecules and ligands located on the leukocytes and endothelial cells of the vessel walls. The neutrophil-endothelial cell adhesion and migration process involves four stages: (1) activation of VEC, (2) activation of neutrophils, (3) binding of neutrophils to inner vessel linings, and (4) transendothelial migration. Neutrophils and monocytes are active in phagocytosis and development of the innate IR. Eosinophils function in defending the body against parasites and are also involved in allergic reactions and chronic inflammation. Basophils are involved in allergic reactions by releasing histamine and heparin when activated via the binding of IgE to membrane Fc receptors.

Monocytes function as phagocytes and secrete a variety of cytokines that affect the function of other cells, especially that of lymphocytes. Monocytes, also referred to as *antigen-processing* (or *presenting*) *cells* (APCs), play a major role in initiating and regulating the adaptive immune response.

Review Questions

Level I

1. Leukocytosis can be defined as an increase in: (Objective 1)
   A. neutrophils, monocytes, and macrophages
   B. neutrophils, eosinophils, erythrocytes, and basophils
   C. neutrophils, eosinophils, basophils, monocytes, and lymphocytes
   D. neutrophils, eosinophils, basophils, monocytes, lymphocytes, and megakaryocytes
2. The hallmark of differentiating myelocytes from promyelocytes morphologically is the visual identification of what in the myelocytes? (Objective 2)
   A. primary granules
   B. secondary granules
   C. loss of nucleoli
   D. pink cytoplasm
3. Primary granules first appear in the: (Objective 2)
   A. myeloblast
   B. promyelocyte
   C. myelocyte
   D. band
4. The eosinophil's primary function is to: (Objective 5)
   A. protect the host from helminth parasites
   B. protect the host from autoimmune destruction
   C. secrete cytokines to attract monocytes to the site of infection
   D. secrete cytokines to attract lymphocytes to the site of infection
5. Leukocyte migration to the tissues is regulated by leukocyte-endothelial cell recognition that requires: (Objective 6)
   A. interaction of adhesion molecules and their receptors
   B. activation of membrane oxidase
   C. leukocyte degranulation
   D. hematopoietic growth factors
6. A(n) _____ has cytoplasm with a ground glass appearance while a _____ contains cytoplasm that is pinkish to clear in color. (Objective 4)
   A. eosinophil; neutrophil
   B. monocyte; lymphocyte
   C. lymphocyte; basophil
   D. monocyte; neutrophil

7. Basophils and mast cells have receptors for which immunoglobulin? (Objectives 3, 5)

   A. IgA

   B. IgG

   C. IgM

   D. IgE

8. The total WBC count for an adult is 13.1 $\times$ 10^9/L with a differential count that reveals 20% eosinophils. This represents a: (Objectives 1, 7, 8, 9)

   A. relative and absolute eosinophilia

   B. relative eosinophilia and normal absolute count

   C. normal relative count and absolute eosinophilia

   D. normal eosinophil count

9. An absolute neutrophilia is most likely to be associated with a(n): (Objectives 1, 10)

   A. allergic response

   B. parasitic infection

   C. bacterial infection

   D. viral infection

10. Routine hematological analysis was performed on a 1-day-old baby. The WBC count was 21.3 $\times$ 10^9/L. This finding represents a(n): (Objectives 1, 11)

    A. normal leukocyte count

    B. absolute leukocytosis

    C. relative leukopenia

    D. absolute leukopenia

11. Which of the following leukocytes are most likely to resemble the morphology of a monocyte? (Objective 4)

    A. neutrophils

    B. basophils

    C. reactive lymphocytes

    D. eosinophils

12. Monocytes function in the innate immune response by their ability to _____ and function in the adaptive immune response by _____ and _____. (Objective 5)

    A. phagocytose; antigen presentation; cytokine secretion

    B. degranulate; erythrophagocytosis; diapedesis

    C. stimulate T cells; secretion of IgM; remove helminths

    D. secrete cytokines; chemotaxis; degranulation

13. Patients with chronic granulomatous disease lack the ability to produce a neutrophilic respiratory burst and often die of bacterial infections. Which phase of phagocytosis is disrupted? (Objective 6)

    A. ingestion of bacteria

    B. bacterial cell killing

    C. neutrophilic degranulation

    D. bacterial cell recognition

14. What would be the major effect on the body of a severe monocytopenia? (Objectives 1, 5)

    A. increased risk of parasitic infections

    B. increased risk of bacterial infections

    C. increased risk of viral infections

    D. decreased risk of allergic reactions

15. If a neutrophil lacked the ability to produce L-selectin, which of its functions or abilities would be disrupted? (Objective 6)

    A. phagocytosis

    B. degranulation

    C. cytokine secretion

    D. margination

Level II

1. The average cell turnover rate for granulocytes and monocytes in the peripheral blood is: (Objective 1)

   A. hours

   B. 24 hours

   C. 8–10 days

   D. 10 years

2. The following cells are found in the granulocytic proliferating pool (mitotic pool) of the marrow: (Objective 1)

   A. pluripotential stem cells

   B. unipotential progenitor cells

   C. monoblasts, myeloblasts, and macrophages

   D. myeloblasts, promyelocytes, and myelocytes

3. Which leukocyte is important in fighting helminth parasitic infection? (Objective 3)

   A. eosinophil

   B. neutrophil

   C. monocyte

   D. basophil

4. An individual who has a mutation in the CD18 gene that results in absence of the $\beta 2$-integrin on the leukocyte membrane will likely have: (Objective 2)
   A. severe allergic reactions
   B. leukocytosis with neutrophilia
   C. life-threatening bacterial infections
   D. a defect in phagocytosis
5. A patient was seen in the ER for symptoms of appendicitis. A complete blood count was ordered. The WBC count was 20×10^9/L and the differential revealed 60% segmented neutrophils, 15% bands, 20% lymphocytes, 4% monocytes, and 1% eosinophils. All other parameters were within the reference interval. These results are most likely due to: (Objectives 1, 4, 5)
   A. release of neutrophils from the bone marrow proliferating/mitotic compartment
   B. release of neutrophils from the bone marrow maturation/postmitotic compartment
   C. a shift in the marginated pool of neutrophils in the peripheral blood compartment
   D. a shift in the circulating pool of neutrophils in the peripheral blood compartment
6. Segmented neutrophils are more capable of egressing from the bone marrow into the peripheral blood than myelocytes because: (Objective 2)
   A. mature neutrophils more easily deform through the small pore diameter in endothelial cells lining the marrow sinusoids
   B. mature neutrophils secrete a cytokine that enables them to adhere to endothelial cells of the marrow sinusoids
   C. there is a higher concentration of mature neutrophils in the bone marrow but myelocytes are rare
   D. only neutrophils possess the secondary granules required for chemotaxis
7. A 30-year-old healthy male needed a CBC as part of his physical examination for purchasing a life insurance policy. He decided to combine his daily 5-mile run with his appointment to get his blood drawn. He ran 3 miles to the laboratory, had his blood drawn, and returned home. His WBC count was increased to 15×10^9/L but all other CBC parameters were normal. What is the most likely explanation for the increased WBC count? (Objectives 1, 5)
   A. He has a bacterial or viral infection.
   B. He has a leukemia with leukemic cells present.
   C. Nucleated RBCs have entered the peripheral blood.
   D. The marginating neutrophil pool entered the circulating pool.
8. If the neutrophil count was determined before and after the run in the patient in question 7, what would be the most likely results? (Objective 5)
   A. absolute neutrophilia before and relative neutrophilia after
   B. relative neutrophilia before and absolute neutrophilia after
   C. neutropenia before and absolute neutrophilia after
   D. increased neutrophils after in comparison to before
9. An absence of the E- and P-selectin receptors on neutrophils results in: (Objective 2)
   A. increased movement of neutrophils from the circulation into the tissues
   B. increased neutrophil response to chemotaxins
   C. arrest of neutrophil rolling on the VEC surface
   D. inability of the neutrophil to interact with the VEC
10. Monocytes contribute to a proper functioning adaptive immune response by: (Objective 3)
    A. processing and presenting foreign antigens to lymphocytes
    B. removing red blood cells from the circulation
    C. removing lymphocytes from the circulation
    D. secreting substances that neutralize inflammatory products of eosinophils
11. A gene defect that reduces the ability of M-CSF to be produced in sufficient quantity would result in a: (Objectives 1, 4)
    A. monocytosis
    B. neutrophilia
    C. monocytopenia
    D. basophilia
12. While neutrophils predominantly function in the tissues, they can function as phagocytic cells in the blood stream during which of the following? (Objectives 3, 4)
    A. when bacteria are present in the blood
    B. during times of intense physical activity
    C. during an allergic response
    D. during a viral infection

13. An adult male presents to the emergency department after experiencing severe nausea and vomiting. The total WBC count for the patient is 7.8×10^9/L with a differential count that reveals 10% monocytes. The laboratory data for this patient represent a: (Objective 5)

    A. relative and absolute monocytosis

    B. relative monocytosis and normal absolute count

    C. normal relative count and absolute monocytosis

    D. normal monocyte count

14. During a severe bacterial infection, the concentration of neutrophils will decrease in which of the following locations? (Objective 1)

    A. the site of infection

    B. the tissues

    C. the proliferating pool

    D. the storage pool

15. A genetic alteration that produces improperly formed Fc receptors on the surface of neutrophils would have which of the following effects? (Objective 3)

    A. The neutrophil would not recognize IgG-coated bacterial cells.

    B. The neutrophil would not be able to phagocytize viral particles.

    C. Activated complement could not bind to the neutrophil receptors.

    D. Phagocytosis could not take place at all.

Companion Resources

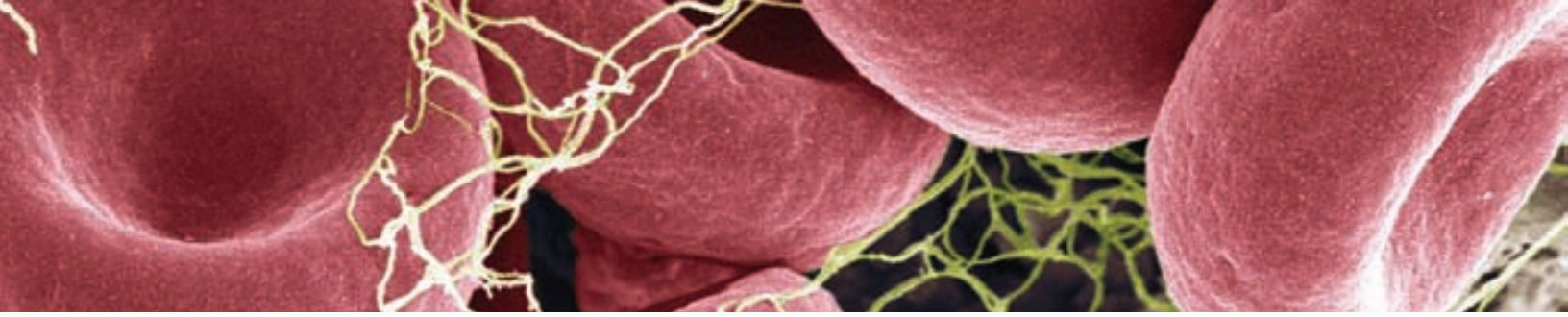
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Wintrobe MM. *Blood, Pure and Eloquent*. New York: McGraw-Hill; 1980:19–22, 428–31.
2. Weaver AD. The development of the knowledge of the leukocyte. *Bull NY Acad Med*. 1954;30:988–92.
3. Berger NA, Rosenblum D. Geriatric hematology. In: Young NS, Gerson SL, High KA, eds. *Clinical Hematology*. Philadelphia: Mosby Elsevier; 2006:944–55.
4. Lim EM, Cembrowski G, Cembrowski M et al. Race-specific WBC and neutrophil count reference intervals. *Int J Lab Hematol*. 2010:32(6):590–97.
5. Beutler B. Innate immunity. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010; 261–72.
6. Abbas AK, Lichtman AH, Pillai S. Innate immunity. In: *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:55–88.
7. Marciniak-Czochra A, Stiehl T, Ho AD et al. Modeling of asymmetric cell division in hematopoietic stem cells—regulation of self-renewal is essential for efficient repopulation. *Stem Cells Dev*. 2009;3:377–85.
8. Khanna-Gupta A, Berliner N. Granulocytopoiesis and monocytopoiesis. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier Churchill Livingstone; 2013:280–91.
9. Smith C. Morphology of neutrophils, eosinophils, and basophils. In: Prchal JT, Kaushansky K, Lichtman MA, Kipps TJ, Seligsohn U, eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:875–82.
10. Baehner RL. Normal phagocyte structure and function. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 4th ed. Philadelphia: Elsevier Churchill Livingstone; 2005:737–62.
11. Clinical and Laboratory Standards Institute. Reference leukocyte (WBC) differential count (proportional) and evaluation of instrumental methods. *Approved Standard*, 2nd ed. CLSI document H-20-A2. Villanova, PA: CLSI; 12. 2007:27(1).
12. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell*. 1994;76:301–14.
13. Rosenzweig SD, Holland, SM. Recent insights into the pathobiology of innate immune deficiencies. *Current Allergy and Asthma Reports*. 2011;5: 369–77.
14. Borregaard N, Boxer LA. Disorders of neutrophil function. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:951–88.
15. Wilson JM, Ziemba SE. Neutrophils fight infectious disease but may promote other human pathologies. *Lab Med*. 1999;30:123–28.
16. Geiszt M, Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem*. 2004;50:51715–18.
17. Jacobsen EA, Taranova AG, Lee NA et al. Eosinophils: singularly destructive effector cells or purveyors of immunoregulation? *J Allergy Clin Immunol*. 2007;119:1313–20.
18. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol*. 2013;13:9–22.
19. Ackerman SJ, Liu L, Kwatia MA et al. Charcot-Leyden crystal protein (galectin-10) is not a dual function galectin with lysophospholipase activity but binds a lysophospholipase inhibitor in a novel structural fashion. *J Biol Chem*. 2002;277:14859–68.
20. Gorczyca W, Sun ZY, Cronin W et al. Immunophenotypic pattern of myeloid populations by flow cytometry analysis. *Methods Cell Biol*. 2011;103:221–66.
21. Wardlaw AJ. Eosinophils and their disorders. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010: 897–914.
22. Powell N, Walker MM, Talley NJ. Gastrointestinal eosinophils in health, disease and functional disorders. *Nat Rev Gastroenterol Hepatol*. 2010;7:146–56.

23. Linch SN, Gold JA. The role of eosinophils in non-parasitic infections. *Endocr Metab Immune Disord Drug Targets*. 2011;11:165–72.
24. Jacobsen EA, Helmers RA, Lee JJ et al. The expanding role(s) of eosinophils in health and disease. *Blood*. 2012;120:3882–90.
25. Wang HB, Ghiran I, Matthaei K et al. Airway eosinophils: allergic inflammation recruited professional antigen-presenting cells. *J Immunol*. 2007;179;7585–92.
26. Jordan MB, Filipovich AH. Histiocytic disorders. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2013:686–700.
27. Befus AD, Denburg JA. Basophilic leukocytes: mast cells and basophils. In: Greer JP, Foerster J, Lukens J et al., eds. *Wintrobe's Clinical Hematology*, 11th ed. Philadelphia: Lippincott Williams & Wilkins; 2004:335–48.
28. Rodewald HR, Dessing M, Dvorak AM et al. Identification of a committed precursor for the mast cell lineage. *Science*. 1996;271:818–22.
29. Pluddemann A, Gordon S. Biochemistry and functions of monocytes and macrophages. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*. 8th ed. New York: McGraw-Hill; 2010:999–1020.
30. Douglas S, Tuluc F. Morphology of monocytes and macrophages. In: Lightman MA, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010: 989.
31. Henriksen K, Karsdal MA, Taylor A et al. Generation of human osteoclasts from peripheral blood. *Methods Mol Biol*. 2012;816:159–75.



8 Lymphocytes

LYNNE WILLIAMS, PHD
KATHLEEN FINNEGAN, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe the function of lymphocytes.
2. Summarize the distribution and state the concentration of lymphocytes in peripheral blood.
3. List the age-related reference intervals for peripheral blood lymphocytes.
4. List causes/conditions associated with an increase or decrease in the absolute numbers of lymphocytes found in the peripheral blood.
5. Outline and describe the development of lymphocytes including distinguishing maturation and morphologic features of cells of the lymphocytic lineage.
6. Describe the morphology of the activated or reactive lymphocyte.
7. Explain immunoglobulin diversity.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Summarize the kinetics of the lymphocytic lineage.
2. Outline and describe the hierarchy of lymphocyte development.
3. Describe and compare T and B lymphocyte developmental stages.
4. Compare and contrast the immunologic features and functions of the various types of lymphocytes found in the peripheral blood.
5. Summarize lymphocyte membrane and molecular characteristics used to differentiate lymphocyte subtypes.
6. Describe the synthesis of immunoglobulin including heavy and light chain gene rearrangement.
7. Differentiate between polyclonal and monoclonal gammopathies and describe each type in relationship to a patient's clinical condition.

Chapter Outline

Key Terms

Adaptive immune response
Antigen-presenting cell (APC)
B-cell receptor (BCR)
Blast transformation
Cell-mediated immunity
Cytokine
Cytotoxic T cell, cytotoxic T lymphocyte (CTC/CTL)
Effector T lymphocyte
Helper T cell (T_H)
Humoral immunity
Immune response (IR)
Immunoblast
Immunoglobulin (Ig)
Innate immune response
Large granular lymphocyte (LGL)
Lymphocytopenia
Lymphocytosis
Lymphokine-activated killer cell (LAK)
Memory cell
Natural killer (NK) cell
Natural killer T (NKT) cell
Plasma cell
Plasmacytoid lymphocyte
Reactive lymphocyte
Recombination-activating gene 1 and 2 (*Rag1*/*Rag2*)
Regulatory T lymphocyte (T_{Reg})
T_H17 cell
T-cell receptor (TCR)
Terminal deoxynucleotidyltransferase (TdT)

Background Basics

In addition to information from previous chapters, having a basic understanding of immunology (immune system and function), biochemistry (proteins, carbohydrates, lipids), and the use of percentages, ratios, and proportions is helpful for understanding the concepts in this chapter. To maximize your learning experience, you should review these concepts from previous chapters before starting this unit of study:

Level I

- Identify cell components and describe their function. (Chapter 2)
- Summarize the function of growth factors, the hierarchy of hematopoiesis, and the concept of the CD nomenclature. (Chapter 4)
- Describe the structure and function of the hematopoietic organs. (Chapter 3)

Level II

- List the growth factors and identify their function in leukocyte differentiation and maturation. (Chapter 4)

CASE STUDY

We will refer to this case study throughout the chapter.

A 6-month-old male infant is seen by his pediatrician for his 6 month checkup. His mother reports that he has had no health problems. A CBC was ordered with the following results: WBC count 14.0 $\times$ 10^9/L; RBC count 4.5 $\times$ 10^{12}/L; Hb 12.5 g/dL: Hct 37.5%; differential: segmented neutrophils 37%; lymphocytes 60%; monocytes 3%.

Evaluate these results as you study this chapter.

OVERVIEW

Lymphocyte precursors develop from the pluripotential hematopoietic stem cell in the bone marrow. They differentiate into several functionally different types of lymphocytes (T, B, natural killer [NK] cells), all of which are involved in an **immune response (IR)**. This chapter is a study of the normal differentiation and maturation of these cells including morphology, concentration, and function. The synthesis and structure of immunoglobulins, lymphocyte receptors, and cell antigens are described with attention to the use of these markers in identifying lymphocyte subpopulations.

INTRODUCTION

For many years after its discovery, the lymphocyte was considered an insignificant component of blood and lymph. Since 1960, however, major advances in immunology have identified lymphocytes as the principal effector cells of the **adaptive immune response**. In contrast to the nonspecific **innate immune response** that involves granulocytes and monocytes and develops rapidly as the body's initial response to pathogens, the adaptive immune response develops more slowly as it generates a response to eliminate specific pathogens. The adaptive immune response involves lymphocytes, monocytes, and other antigen-presenting cells and results in immunologic memory by which the lymphocytes are primed to rapidly respond to antigens on subsequent encounters. The two types of adaptive immune responses are **humoral immunity** and **cell-mediated immunity**, which function to eliminate different types of molecules that can be infectious (e.g., microbes) or simply considered foreign to the body (e.g., cancer cells).[1]

Lymphocytes include subpopulations of cells with various origins, life spans, and function. These cells show more morphologic variation than other WBCs. The lymphocyte's life can span several years or the cell can die within hours or days. The primary functions of lymphocytes are to recognize and react with specific antigens, work with macrophages to eliminate pathogens, and provide long-lasting immunity to previously encountered pathogens.

There are three types of lymphocytes that are morphologically identical but immunologically and functionally diverse: T lymphocytes, B lymphocytes, and natural killer (NK) cells. T lymphocytes and B lymphocytes are the major cells of the adaptive immune response. The NK cells have characteristics distinctly different from those of T and B lymphocytes and are effector cells of innate immunity. B lymphocytes are the primary effector cells for the humoral immune response (i.e., the production of antibodies). To accomplish effective humoral immunity, the B lymphocyte must be activated and differentiate to a plasma cell. T lymphocytes are primarily involved in cell-mediated immunity (CMI), which requires interaction among macrophages, T lymphocytes, and antigens. CMI is independent of antibody production by B lymphocytes. B and T lymphocytes consist of several distinct subpopulations with different phenotypic markers and functions. There are two subsets of B cells (B-1 and B-2) and at least three important functional subsets of T lymphocytes: helper

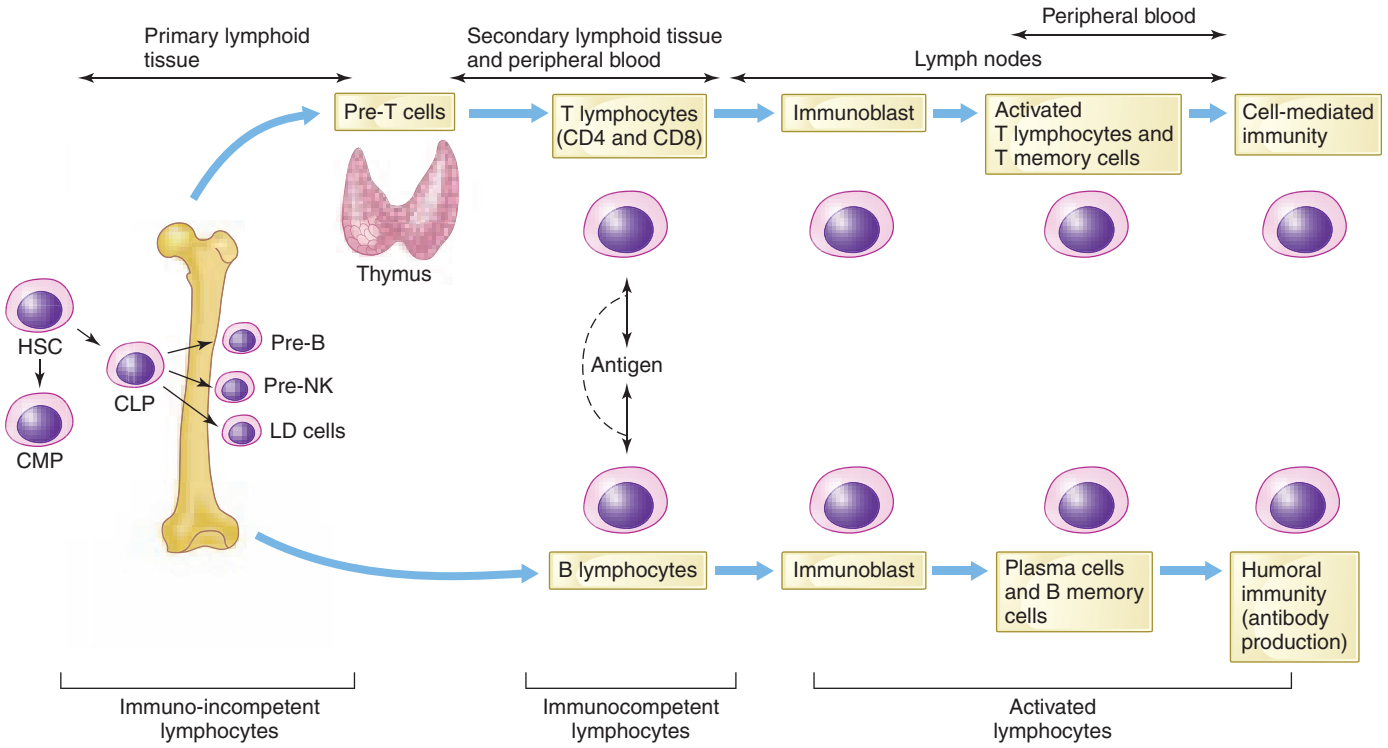
T lymphocytes (T_H), cytotoxic T lymphocytes (CTLs), and regulatory T lymphocytes (T_{Reg}). Effective immunocompetence (ability to generate an adaptive immune response) depends on a balance and interaction between B and T lymphocytes.

LYMPHOPOIESIS

The lymphoid lineage arises from the pluripotential hematopoietic stem cell (HSC) found in the bone marrow. The HSC gives rise to committed progenitor cells: the common lymphoid progenitor cell (CLP) and the common myeloid progenitor cell (CMP) (Chapter 4). The CLP differentiates and matures under the inductive influence of selective microenvironments and cytokines into lymphocytic subpopulations of T cells, B cells, NK cells, and some dendritic cells (Figure 4-3). Commitment to the specific lymphocytic lineage (T, B, NK) is the result of specific transcription regulators (transcription factors) that influence a CLP to assume a B-cell, T-cell, or NK-cell fate. Notch-1, a cell surface molecule, interacts with the transcription factor GATA-3 to commit lymphoid precursors to the T lineage, whereas three separate transcription factors, EBF, E2A, and Pax-5 interact to induce commitment to the B lineage.[2]

Like production of other hematopoietic lineages, lymphopoiesis occurs in the fetal liver before birth and largely in the bone marrow after birth. Fetal liver–derived lymphoid precursors give rise to lymphocytes with distinctly different characteristics than those derived from the bone marrow. Fetal liver–derived B cells are mainly B-1 cells and fetal liver–derived T cells are primarily γδ T cells (see the section "Lymphocyte Function"). These B and T cells generated during fetal development generally have less diverse antigen receptors than cells derived from bone marrow HSCs.[2]

T and B lymphopoiesis can be divided into two distinct phases: antigen-independent lymphopoiesis and antigen-dependent lymphopoiesis (Figure 8-1 ■). Antigen-independent lymphopoiesis takes place within the primary lymphoid tissue (liver in the fetus; bone marrow for B cells, thymus for T cells in the adult). Antigen-independent lymphopoiesis begins with the CLP and results in the formation of immunocompetent T and B lymphocytes (referred to as *virgin* or *naïve lymphocytes* because they have not yet reacted with antigens). These cells exit the primary lymphoid tissue and migrate to



■ **FIGURE 8-1** Lymphocytes originate from the common lymphoid progenitor cell/CLP (derived from the pluripotential hematopoietic stem cell/HSC) in the bone marrow. Lymphocyte progenitors that mature in the thymus become T lymphocytes, and those that mature in the bone marrow become B lymphocytes, natural killer cells (NK), or lymphoid-derived dendritic cells (LD). Three morphologic stages can be identified in this development to T and B cells: lymphoblast, prolymphocyte, and lymphocyte. On encounter with antigen, these immunocompetent T and B lymphocytes undergo blast transformation, usually in the lymph nodes, to form effector lymphocytes. The B lymphocytes eventually emerge as plasma cells. Effector T lymphocytes, however, are often morphologically indistinguishable from the original T lymphocytes. The recognizable morphologic stages of blast transformation include reactive lymphocytes, immunoblasts, plasmacytoid lymphocytes (B cells), and plasma cells (B cells). Flow cytometry indicates that some morphologic stages could represent several stages of immunologic maturation.

secondary lymphoid tissue (spleen, lymph nodes, gut-associated lymphoid tissue) where the antigen-dependent phase of lymphopoiesis takes place. Antigen-dependent lymphopoiesis begins with the recognition of and the interaction with antigens by specific antigen receptors on the surface of the immunocompetent T and B lymphocytes (**T-cell receptors [TCRs]** and **B-cell receptors [BCRs]**). These receptors are not encoded by conventional genes but are derived from rearrangement of DNA segments in the respective gene loci to create billions of different versions of the basic receptor.[3] As a result these receptors are highly variable and pathogen-specific. Interaction with antigen results in the formation of effector T and B lymphocytes, which undergo subsequent changes in phenotype and functional capacity. These effector cells mediate a pathogen-specific adaptive immune response.

Effector T lymphocyte subsets include cells responsible for cell-mediated cytotoxic reactions (**cytotoxic T lymphocytes [CTLs]** or **cytotoxic T cells [CTCs]**); cells that provide helper activity for B cells, macrophages, and other T cells (**helper T cells [T_H]** with subsets T_H1, T_H2, T_H17); and cells that function to suppress other T-cell immune responses (**regulatory T cells [T_{Reg}]**). There are two B lymphocyte subsets, B-1 (a minor component of B lymphocytes) and B-2 (the majority of B cells in the blood and lymphoid tissues). Each mature B lymphocyte makes a specific antibody targeted against a specific triggering antigen by rearranging its immunoglobulin genes. They also can concentrate and present antigens to T cells and are the precursors of immunoglobulin-secreting plasma cells. The NK cell is a form of cytotoxic lymphocyte that functions as part of the innate immune system. NK cells play a key role in the cytolysis of both tumor and pathogen-infected cells. They do not rearrange or express T-cell receptor genes or B-cell immunoglobulin genes and thus do not express antigen-specific receptors.

☑ CHECKPOINT 8-1

Describe the subsets of T cells and B cells derived from the CLP.

LINEAGE DIFFERENTIATION

The cells of the innate immune system (neutrophils, monocytes, macrophages) recognize pathogens using a limited number of receptors called *pattern recognition receptors (PRRs)*. PRRs are the protein products of inherited genes and function to recognize molecules unique to human pathogens (*pathogen-associated molecular patterns [PAMPs]*) (Chapter 7). In contrast, in the adaptive immune response lymphocytes recognize pathogens using receptors (BCRs/TCRs) that are not inherited as functional genes but are made during lymphopoiesis. It has been estimated that an individual has $\geq 10^7$ different B-cell and T-cell receptors, each expressed by a small subset of lymphocytes.[2] Only those cells bearing receptors that recognize a particular pathogen will be activated to effector cells when encountering that particular pathogen. The molecule, virus particle, or cell structure that is recognized by the BCR/TCR is called its corresponding *antigen*, and the receptors themselves are thus also referred to as the *antigen receptors* of the lymphocytes.

The diversity of the BCRs/TCRs ($\geq 10^7$) does not require a large number of distinct antigen receptor genes. Rather, diversity arises due to a unique genetic process in which different parts of the polypeptides that make up the receptors are encoded by separate gene segments called *V*, *D*, and *J*. The gene regions for the BCR and TCR polypeptides have arrays of many different V, D, and J gene segments. The chromosomal regions containing these V, D, and J segments are in a disconnected form (called the *germline configuration*) and cannot be transcribed and translated into protein in most of the cells of the body. During lymphopoiesis, however, one of each segment (as appropriate) is brought together by breaking and rejoining the DNA and eliminating intervening regions, resulting in a functional gene. DNA cleavage is mediated by two lymphocyte-specific proteins (**recombination-activating gene 1** and **recombination-activating gene 2 [*Rag1*/*Rag2*]**), which together are called the *V(D)J recombinase*. The lymphoid-specific enzyme **terminal deoxynucleotidyltransferase (TdT)** randomly adds bases to the broken DNA ends, contributing to additional receptor diversity. The broken DNA ends are ligated by the same double-stranded break repair process that all cells use to repair damaged DNA. This process of gene rearrangement is known as *somatic recombination* (or *VDJ rearrangement*) and is the principle source of the great diversity of pathogen receptors that exist for lymphocytes.[2] This process also plays a role in defining populations of precursor lymphocytes during the first phase of lymphopoiesis in the primary lymphoid organs.

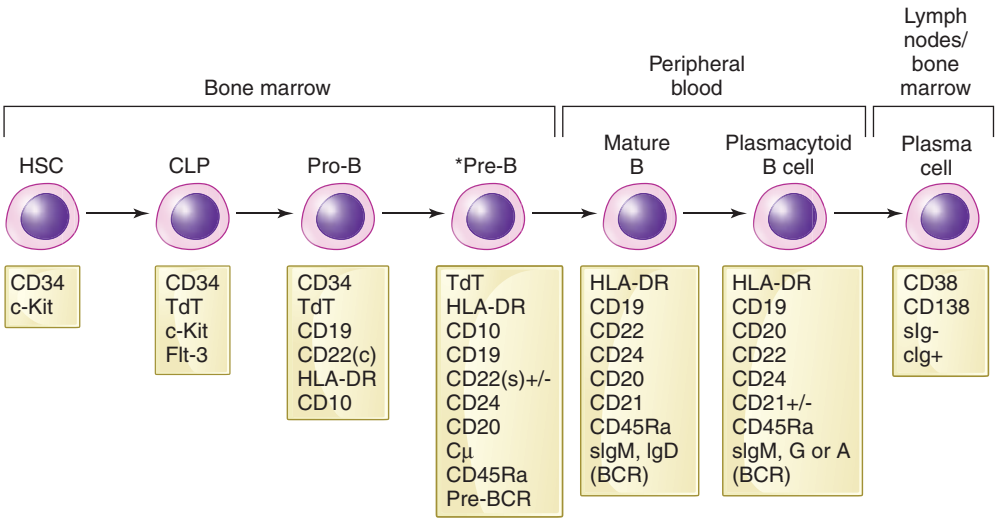
B Lymphocytes

B lymphocyte precursors arise in the bone marrow from the CLP derived from hematopoietic stem cells. The B lymphocyte precursors undergo the antigen-independent phase of their development entirely within the bone marrow and exit as virgin or naïve B lymphocytes. B lymphocytes compose 15–30% of peripheral blood lymphocytes.

B Lymphocyte Membrane Markers

B lymphocytes can be identified and differentiated from T lymphocytes by the presence of surface membrane immunoglobulin and CD antigens/markers. Surface membrane immunoglobulin and CD markers can be detected by fluorescein-conjugated antisera to the proteins, using flow cytometry (Chapter 40).

Membrane markers are present at various stages in B lymphocyte development. B lymphocytes are identified using a panel of antibodies to surface antigens that corresponds to the stage of the cell's differentiation (Figure 8-2 ■). CD markers expressed by B lymphocytes and their precursors include CD10, CD19, CD20, CD21, CD22, CD24, and CD38. The CD19 antigen is considered a "pan-B" antigen because it is found on the earliest B lymphocyte and is retained until the latest stages of activation. The CD10 marker, also known as the **c**ommon **a**cute **l**ymphoblastic **l**eukemia **a**ntigen (CALLA), was originally believed to be a specific marker of leukemia cells in acute lymphoblastic leukemia. It is now known that CALLA is present on a small percentage (<3%) of normal bone marrow cells and is found only on early B lymphocyte precursors and disappears as cell maturation occurs.



■ **FIGURE 8-2** Immunologic maturation of the B lymphocyte from the pluripotential hematopoietic stem cell (HSC) to the plasma cell. Specific antigens (CD) that appear sequentially on the developing cell can define each maturation stage. The drawing shows selected differentiation antigens. Stem cells, pro-B lymphocytes, and pre-B lymphocytes are normally found in the bone marrow. The mature B lymphocyte is found in the peripheral blood. When stimulated by antigens, the B lymphocytes undergo maturation to plasma cells in the lymph nodes or bone marrow.

Cμ = cytoplasmic μ chains; sIg = surface membrane immunoglobulin; cIg = cytoplasmic immunoglobulin; CD = cluster of differentiation; BCR = B-cell receptor; c-Kit = stem cell factor receptor, also called CD117.

B Lymphocyte Antigen Receptor (BCR)

B cells recognize and interact with specific antigens via their antigen-specific BCR (Figure 8-3a ■). The BCR consists of an immunoglobulin (Ig) molecule identical to that produced by the mature B lymphocyte/plasma cell. The complete BCR includes the immunoglobulin molecule with two accessory molecules, Igα and Igβ, which function as signaling molecules during B-cell activation.[2]

Immunoglobulin

Immunoglobulin (Ig) is a unique molecule produced by B lymphocytes and plasma cells. Ig, also known as *antibody*, consists of two pairs of polypeptide chains: two heavy and two light chains linked together by disulfide bonds (Figure 8-4 ■). The number and arrangement of these bonds are specific for the various Ig classes and subclasses. There are five types of heavy chains, α, δ, ϵ, γ, and μ, which determine the class of the antibody (IgA, IgD, IgE, IgG, IgM, respectively). Although each B-cell precursor has two sets of heavy chain genes (one on each chromosome 14), only one encodes the heavy chain protein in any given cell. Thus, within a given Ig molecule, the two heavy chains are always identical. There are two types of light chains, κ and λ, and as with the heavy chains, the two light chains within an Ig molecule are always identical. Either κ- or λ-chain can be found in association with any of the various heavy chains, forming the five classes of antibodies. Each of the classes and subclasses of Ig has distinct physical and biologic properties (Web Table 8-1).

Each heavy and light Ig chain consists of a variable region and a constant region. The constant region is the same for all antibodies within a given class or subclass, but the variable region in each Ig molecule differs. The constant region mediates effector functions such as complement activation. Together, the variable regions of the light (V_L) and heavy (V_H) chains determine the antibody-combining site. Somatic recombination (or gene rearrangement) of gene segments of the heavy chain and light chain variable regions allows a diverse repertoire of Ig specificity for antigens.

Immunoglobulin Gene Rearrangement

The Ig molecule that will eventually be produced by mature B cells and plasma cells serves as part of the BCR of B cells and undergoes the VDJ rearrangement discussed previously. The initiation of Ig gene rearrangement is one of the earliest features allowing the cell to be recognized as a B-cell precursor and is a marker of commitment to the B-cell lineage. Heavy chain and light chain gene rearrangement occurs at different stages of B-cell development (see section "B Lymphocyte Developmental Stages"). Rearrangement of the heavy chain locus involves random selection of a coding sequence from each of three groups of DNA segments (V, D, J) and recombination to form a variety of unique antibody specificities. The first step is splicing a D and J segment (DJ); subsequently a V segment is added to the fused DJ, forming the VDJ sequence that codes for the variable region of the heavy chain (Figure 8-5 ■). The κ and λ light chain also rearrange gene segments, but light chain–variable gene segments include only a V and J region (no D region).

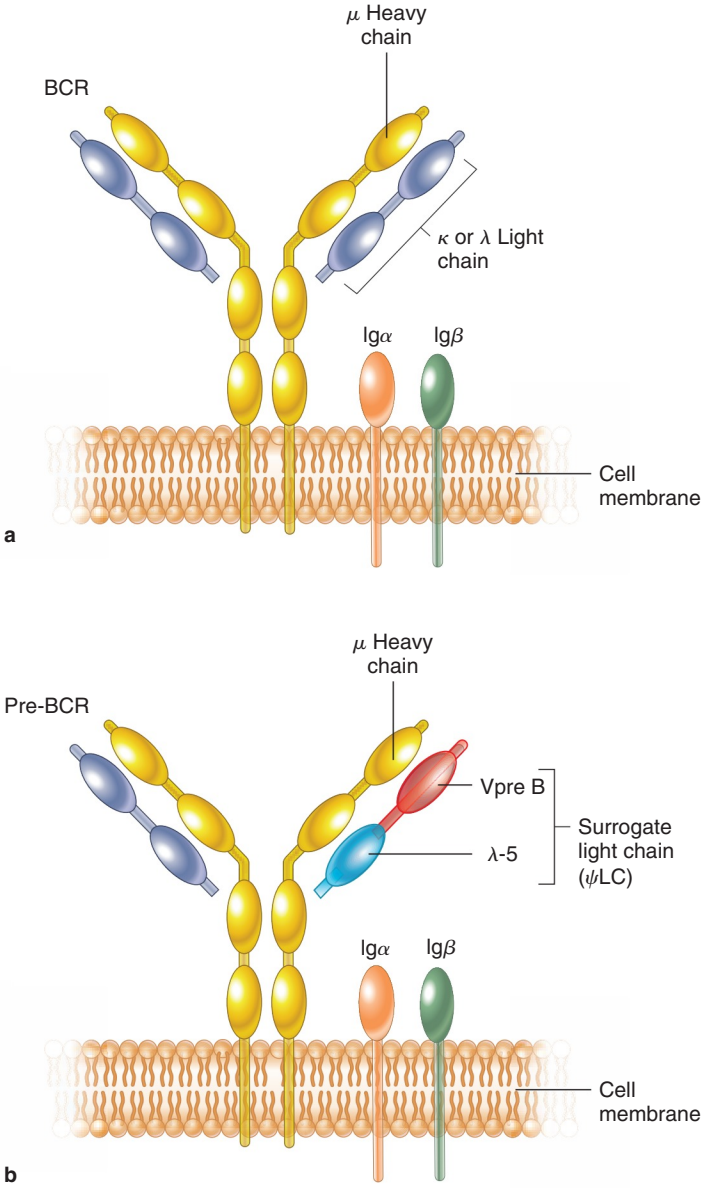


FIGURE 8-3 **(a)** BCR. The B-cell receptor (BCR) consists of an immunoglobulin molecule with two heavy chains (HC) and two light chains (LC) complexed with accessory molecules Igα and Igβ, needed for cell signaling. **(b)** Pre-BCR. During development, before the cells are fully mature, a "pre-BCR" is made, using a surrogate LC composed of two molecules (Vpre B and λ-5) complexed with a μ HC and the Igα and Igβ accessory molecules.

B Lymphocyte Developmental Stages

Differentiation of the developmental stages for both B and T lymphocytes is based on the appearance of specific molecular changes as well as membrane molecules. Distinct stages of B cells are defined using monoclonal antibodies to identify the presence of CD markers on the cell surface as well as molecular evidence of Ig gene rearrangement. Some CD markers appear at a very early developmental stage of the cell and disappear with maturity; other CD markers appear on more mature cells (Figure 8-2).

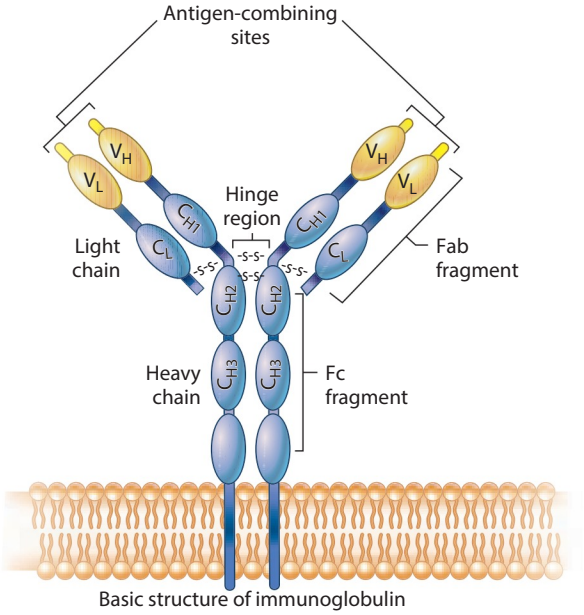


FIGURE 8-4 Schematic drawing of an IgG molecule. The four peptide chains include two light and two heavy chains. Disulfide bonds between the chains are indicated by -s-s-. The variable domains are indicated by V and the constant regions by C. The variable regions have variable amino acid sequences depending on the antibody specificity, and the constant regions have a constant sequence among immunoglobulins of the same class. The two heavy chains (H) determine the class of immunoglobulin, in this case for IgG. The two light chains can be either κ or λ.

Early antigen-independent stages of B lymphopoiesis occur in the bone marrow where marrow stromal cells provide a specialized microenvironment for B cells at various stages of maturation. Cytokines important in the early phases of B-cell development include SCF, Flt-3 ligand (FL), and SDF-1.[4]

The earliest committed B-cell precursor is the *pro-B cell* (Figure 8-1).[5] It is characterized by the presence of CD34 (an early hematopoietic cell marker), CD19 (pan-B marker), CD10 (CALLA), and TdT. During the pro-B stage, heavy chain rearrangement of the μ heavy chain of IgM takes place. Only about half of the total number of pro-B cells successfully makes a functional heavy chain; the other half dies of apoptosis in the marrow. When a successful μ heavy chain rearrangement has been completed, free cytoplasmic μ chains (Cμ) can be found and the cell becomes an early (large) pre-B cell. *Pre-B cells* are characterized by the loss of CD34, persistence of CD19 and CD10 (CALLA), and the appearance of CD20 and CD24. Pre-B cells produce a "pre-BCR" on the cell surface consisting of the μ heavy chain complexed with a surrogate light chain (because true light chains are not yet being produced), and the accessory molecules Igα and Igβ (Figure 8-3b). Once formed, the pre-BCR delivers to the cell signals that promote survival, proliferation, and continued maturation.[2] Toward the end of the pre-B-cell stage, light chain gene rearrangement occurs; it is characterized by loss of TdT and persistence of CD-19, -20, and -24. About 85% of the pre-B-cell population makes a successful rearrangement of the light chain.[6]

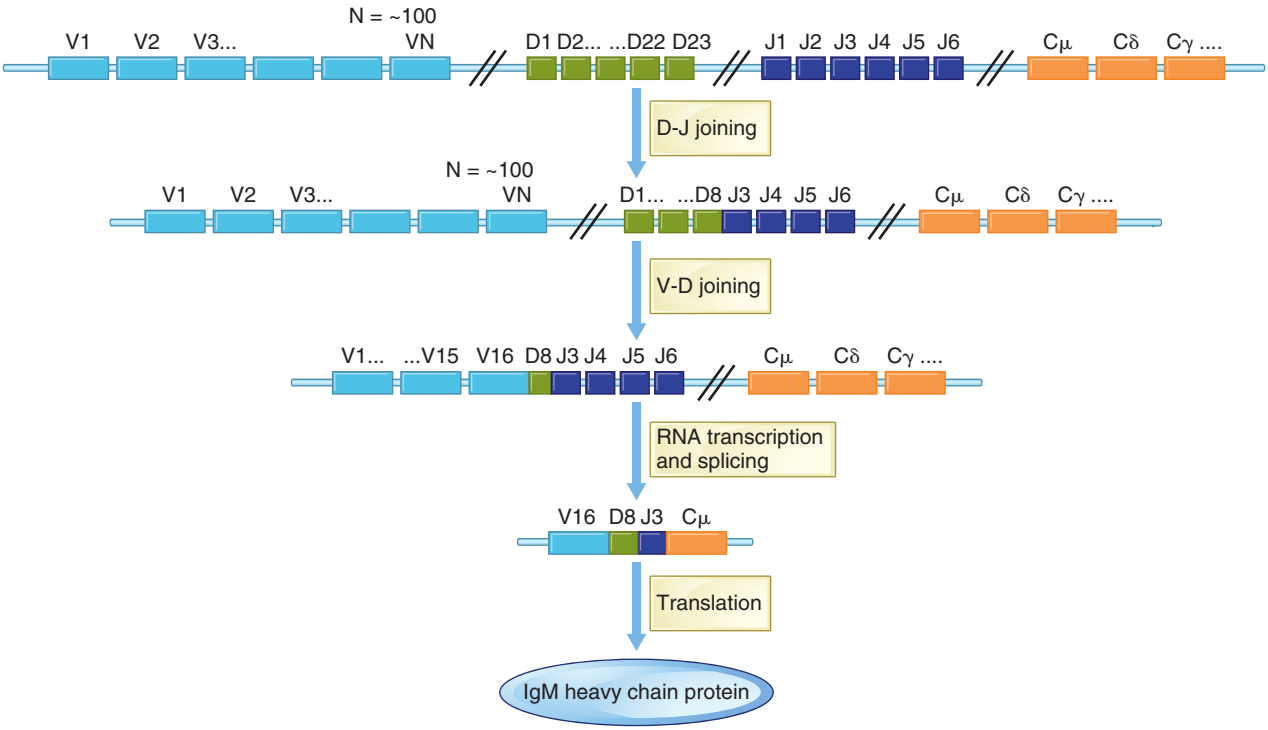


FIGURE 8-5 During B-cell differentiation, the immunoglobulin heavy chain (HC) gene rearranges to produce a unique coding sequence that determines antibody specificity. This occurs through a process of splicing and deletion whereby 1 of 23 diversity (D) regions is juxtaposed with 1 of 6 joining (J) regions and then with 1 of ~100 variable (V) regions. Finally, constant (C) region splicing determines antibody isotype (IgM, IgD, IgG, or IgA). In this example, V, D, J, and Cμ segments are sequentially spliced together to generate a nucleic acid sequence that encodes IgM HC proteins. These HC proteins complex with κ or λ light chain proteins (that are also encoded by rearranged genes) to produce a functional antibody molecule. Each developing B cell has different Ig gene rearrangements, so a population of normal B cells is characterized by polyclonal Ig genes. The diversity of these genes and their encoded antibodies permits immune recognition of many different antigens.

When successful rearrangement of the light chain locus is complete and the cell expresses either a $\kappa\mu$ or a $\lambda\mu$ IgM, the molecule is transported to the cell surface to form a functional BCR (surface IgM [sIgM] plus Igα and Igβ). The cell is now an *immature B cell*; it is positive for CD-19, -20, -21, -22, and -24. The immunocompetent but naïve *mature B cell* is characterized by the dual expression of both μ (IgM) and δ (IgD) BCR on the cell surface. If immature or mature B cells recognize and bind self-antigen in the bone marrow with high avidity, the cells either undergo a process called *receptor editing* (in which B cells alter their light chain to eliminate self-reactivity), or cell death (apoptosis) occurs. During antigen-independent lymphopoiesis in the marrow, the processes of both proliferation and apoptotic cell death are significant. This eliminates those cells with unsuccessful or nonfunctional Ig gene rearrangement as well as those that produce autoreactive antigen receptors.

B lymphocytes are not capable of reacting with antigens until they develop both IgM and IgD on their surface (i.e., mature B-cell stage). The immunocompetent but naïve cells leave the marrow, circulate through the blood, and enter the peripheral lymphoid tissues where the antigen-dependent phase of B-cell development occurs. Mature B cells enter the germinal centers (follicles) of lymph nodes, the germinal centers of the spleen, and other secondary lymphoid tissue where they can undergo further differentiation into effector B cells (plasma cells) in response to encounters with specific antigens.

☑ CHECKPOINT 8-2

What characteristics differentiate an immature and a mature B lymphocyte?

After encountering the antigen recognized by the specific BCR, some activated B cells undergo *isotype switching* of the constant region of the heavy chain gene, converting to IgG, IgA, or IgE BCR. Most mature B lymphocytes possess only one class of immunoglobulin on their membrane.

If B cells fail either to enter a lymphoid follicle or to encounter and be stimulated by specific antigens, the cells die (by apoptosis) within several days to several weeks. However, encounter with specific antigen causes the naïve B cell to undergo proliferation (clonal expansion) and differentiation into plasmacytoid lymphocytes and finally into antibody-secreting plasma cells. Some of the progeny of this clonal

expansion will become long-lived **memory cells**, which have the capacity to rapidly respond to subsequent exposures to the same antigen.

B-Cell Subclasses

Two subclasses of B lymphocytes are detectable in adults. B-1 lymphocytes represent a minority subset of B cells (only ~5% of the total B cells) and are produced from the HSCs that are primarily active in the fetal liver during prenatal hematopoiesis. B-1 lymphocytes are distinguished from other B cells by a number of characteristics: (1) they have a limited diversity of antigen receptors, (2) they are found primarily in pleural, peritoneal, and mucosal sites, and (3) they recognize common bacterial polysaccharides and other carbohydrate antigens, and are thought to provide defense against environmental flora. They are the primary source of the "naturally occurring" anti-A and anti-B antibodies characteristic of the ABO blood group system. The population of B-1 cells is maintained in adults largely by the division of existing B-1 cells.

The majority of B lymphocytes are sometimes called B-2 cells. B-2 lymphocytes are the major B cells produced after birth and are found primarily in the circulation and secondary lymphoid tissues.

☑ CHECKPOINT 8-3

What cells produce immunoglobulin? Describe the structure of an immunoglobulin molecule.

T Lymphocytes

Bone marrow lymphoid precursor cells migrate to the thymus (primary lymphoid organ) where they proliferate and differentiate to acquire cellular characteristics of T lymphocytes. The thymic environment provides stimuli required for the proliferation and maturation of developing T cells. These stimuli include direct physical interaction with cortical and medullary epithelial cells and dendritic cells as well as cytokines including IL-7, SCF, FL, and SDF-1.[4] Developing T cells in the thymus are called *thymocytes*. Lymphopoiesis at this stage correlates to the antigen-independent stage of T lymphocyte development.

The historic viewpoint was that the thymus functions in T lymphopoiesis primarily during fetal life and the first few years after birth and that the T lymphoid system was considered to be nearly fully developed at birth. If the thymus does not properly develop in the fetus (DiGeorge syndrome; Chapter 22), the result is the absence of T lymphocytes as well as severe impairment of adaptive immunity. Surgical removal of the thymus after birth, however, does not significantly impair immunologic defense. By one year after birth, the lymphoid tissue in the thymus begins to be gradually replaced by fat (called *involution of the thymus*).[2,7] Although thymic function declines significantly with age (by 5-fold at age 35 years), the T lymphocyte pool continues to be replenished by the residual functioning thymus throughout life.[8]

Major Histocompatibility Complex

Effective functioning of T lymphocytes in the adaptive IR requires interaction with "histocompatible" macrophages, dendritic cells, B lymphocytes, and other infected host cells. This recognition and interaction between T cells and other cells of the body is achieved via specialized proteins, the major histocompatibility complex (MHC) molecules or antigens. The MHC antigens in humans are called the *human leukocyte antigens (HLAs)* (Table 8-1 ★). The MHC locus on chromosome 6 contains two types of HLA genes important for T-cell function, Class I and Class II, which encode two groups of structurally distinct proteins. There are three major Class I gene loci—*HLA-A*, *HLA-B*, *HLA-C*—and three Class II gene loci—*HLA-DP*, *HLA-DQ*, and *HLA-DR*. Class I MHC molecules (HLA-A, HLA-B, HLA-C proteins) are found on essentially all nucleated cells. MHC Class II molecules (DP, DQ, DR proteins) are found on B lymphocytes, dendritic cells, macrophages, and activated T lymphocytes. The HLA molecules function to present antigens to T lymphocytes and must be recognized by the T cell (i.e., "histocompatible") to elicit an immune response (see "Lymphocyte Function"). The MHC Class III genes code for components of the complement system and some selected cytokines.

T Lymphocyte Membrane Markers

T lymphocytes possess receptors for the Fc portion of IgG (CD16). Additionally, CD surface markers used to differentiate developmental stages of T precursor cells include CD2, CD3, CD5, CD7, CD25, and CD4 and/or CD8.

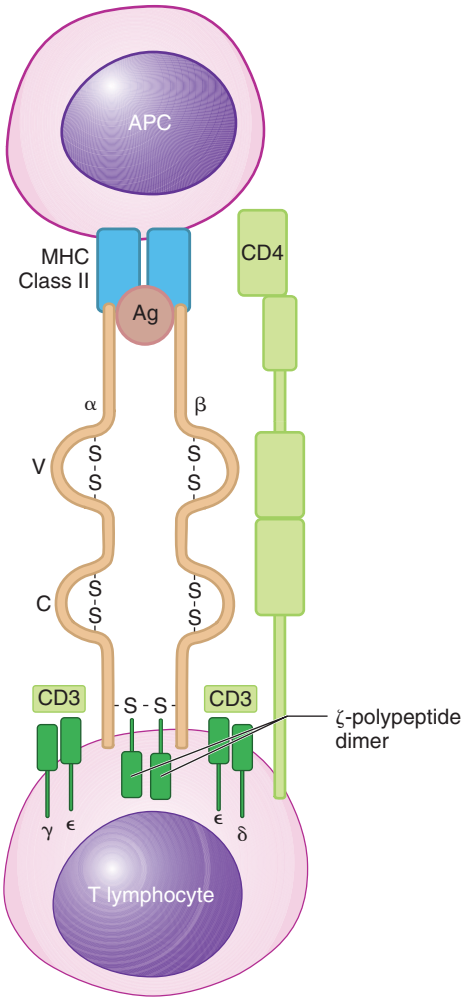
T Lymphocyte Antigen Receptor

The T lymphocyte antigen receptor, also called the T-cell receptor (TCR), differs from the BCR in that it is a heterodimer of two peptide chains linked by a disulfide bond (Figure 8-6 ■). Two subsets of T cells are defined by expression of distinct TCR polypeptides: TCR with γ and δ chains ($\gamma\delta$ T cells) and TCR with α and β chains ($\alpha\beta$ T cells). More than 90% of T lymphocytes are $\alpha\beta$ T cells.[9,10] The organization of the TCR chains is similar to that of the BCR immunoglobulin chains. Each TCR chain is composed of a variable region that recognizes and binds an antigen and a constant region that anchors the TCR to the cell membrane. Similar to the H and L chains of the BCR, the variable (V) region is made from three DNA segments—V, D, and J in the β and δ chains and V and J segments in the α and γ chains. A complete V region (i.e., VDJ or VJ) is joined to a constant (C) segment to form the individual TCR chains. The random rearrangement and recombination of various V, D, and J segments and subsequent joining with a C segment provide the antigenic diversity of TCR, allowing the T lymphocyte to recognize many different antigens.

The $\alpha\beta$ and $\gamma\delta$ TCR are expressed in the T-cell membrane as a molecular complex with several other transmembrane molecules, including the CD3 complex (consisting of three polypeptides, γ, δ, and ϵ, in the form of δ:ϵ and γ:ϵ heterodimers) and a ζ-chain homodimer (CD247) (Figure 8-6). These auxiliary molecules (CD3 and CD247) with the $\gamma\delta$TCR or $\alpha\beta$TCR mediate intracellular signaling when antigen binds to the TCR.[11]

★ **TABLE 8-1** Major Histocompatibility Complex Antigens

Class I	Class II (Ia antigens)	Class III
HLA-A	HLA-DP	Complement components
HLA-B	HLA-DQ	Cytokines
HLA-C	HLA-DR	



■ **FIGURE 8-6** The T lymphocyte has an antigen receptor (TCR) on its surface that is composed of two peptide chains linked by a disulfide bond. This receptor is expressed in a complex with CD3 ($\gamma\epsilon$ and $\delta\epsilon$ polypeptide dimers) and a ζ polypeptide dimer. This TCR is in close proximity to a major histocompatibility complex (MHC)–restricted co-receptor (CD4 or CD8) that recognizes the appropriate MHC molecule on antigen-presenting cells (APCs). The helper T lymphocyte co-receptor (CD4) shown here recognizes Class II MHC molecules, and the cytotoxic T lymphocytes co-receptor (CD8) recognizes Class I MHC molecules.

T Lymphocyte Developmental Stages

Commitment to the T-cell lineage depends on cytokines produced by the thymic microenvironment including IL-7 and tumor necrosis factor-α (TNF-α). Although the exact lymphoid progenitor cell that migrates to the thymus is unclear, acquisition of the cell surface marker CD1 identifies a precursor cell committed to T lineage differentiation.[12,13] Differentiation of the various developmental stages for T lymphocytes parallels that for B lymphocytes and is defined by the presence of CD markers on the cell surface as well as molecular evidence of TCR gene rearrangement (Figure 8-7 ■).The major developmental stages are pro-T cell, pre-T cell (double negative/DN), double positive (DP) and single positive (SP) thymocyte, and T cell.

The earliest committed T-cell precursor, a *pro-T cell*, is CD34+ and contains germline configuration of the TCR α-, β-, γ-, and δ-gene loci. These cells are also CD25+ (IL-2 receptor α-chain), CD44+ (thymic homing receptor), TdT+, and lack CD4 and CD8 (i.e., "double negative" cells). During the pro-T stage, rearrangement of the β-chain locus occurs. If the rearrangement is successful, the β-chain is expressed in the cytoplasm (cβ) toward the end of the pro-T stage. These immature thymocytes are found in the cortex of the thymus and migrate through the cortex to the medulla as maturation proceeds.

During the *pre-T-cell* stage, the TCR β-chain appears on the cell surface and is linked with a surrogate α-chain (preT_α), the CD3 complex, and ζ chains to form the pre-TCR. Signals from the pre-TCR support survival and proliferation (clonal expansion) of the pre-T thymocytes, initiate recombination of the TCR α-chain, and induce expression of both CD4 and CD8 forming *double positive (DP) thymocytes* (late pre-T cell).

When a functional α-chain has been produced, it replaces the surrogate α-chain and pairs with the β-chain to form the $\alpha\beta$TCR that associates with the CD3 complex and ζ-chains. Subsequently, the DP thymocyte that successfully undergoes selection proceeds to differentiate into either single positive CD4+ CD8− or CD4− CD8+ T cells. At this point, the cells are immunocompetent but naïve T cells.

Thymocytes must survive two selection processes during development in the thymus. Mature T cells must be able to recognize foreign peptides presented by "self" MHC molecules. DP thymocytes expressing $\alpha\beta$TCR encounter self-peptides displayed by self-MHC molecules on cortical epithelial cells (the only peptides normally present in the thymic cortex). Thymocytes with TCR that recognize and bind *weakly* to self-antigens presented by self-MHC are stimulated to survive (positive selection). Cells whose TCRs fail to recognize self-MHC molecules die by apoptosis. Thymocytes whose TCRs bind *strongly* to self-antigens are deleted by apoptosis (negative selection) or differentiate into T_{Reg} cells (which function to prevent autoimmune reactions). Positive selection ensures that T cells are self-MHC restricted whereas negative selection ensures that they are tolerant (e.g., do not respond) to most self-antigens. Intrathymic proliferation and death for potential T lymphocytes is high with about 95% of the cells produced undergoing apoptosis. Consequently, only a small portion of T precursor cells developing in the thymus leave as immunocompetent, naïve T lymphocytes.

The differentiation into single positive CD4+ or CD8+ thymocytes is determined by whether an individual thymocyte's TCR recognizes antigen bound to MHC Class II or MHC Class I molecules, respectively. This is primarily mediated by medullary epithelial cells as well as macrophages and dendritic cells.

Single positive cells leave the thymus and enter the circulation to complete final maturation. In the periphery, encounters with the antigen specifically recognized by the unique TCR result in activation and generation of appropriate *effector T lymphocytes* and memory T cells (antigen-dependent phase of lymphopoiesis). CD4+ CD8− cells become helper T cells (T_H), and CD4− CD8+ cells become cytotoxic T cells (CTCs).

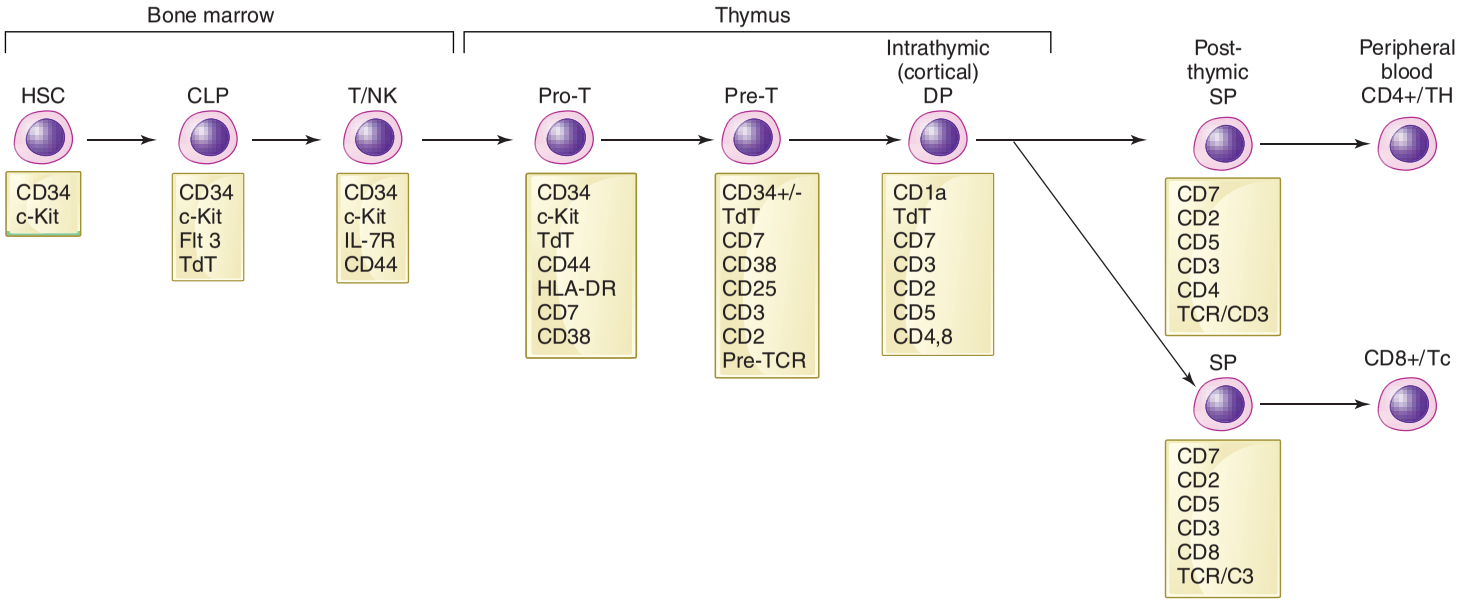


FIGURE 8-7 Immunologic maturation of the T lymphocyte from the common lymphoid progenitor cell (CLP) to the peripheral blood CD4+ and CD8+ lymphocytes, showing selected identification markers. Monoclonal antibodies and molecular studies for TCR gene rearrangements have identified at least four intrathymic stages of maturation before the cells are released to the peripheral blood as mature T lymphocytes. These include pro-T cells, pre-T cells, double positives (DP), and single positives (SP). Differentiation into either CD4+ or CD8+ lymphocytes occurs at the last intrathymic stage of maturation.

CD = cluster of differentiation; TCR = T-cell receptor; TdT = terminyldeoxynucleotidyltransferase; IL7R = interleukin 7 receptor; c-Kit = stem cell factor receptor, or CD117

T Lymphocytes

T lymphocytes constitute about 70% of total peripheral blood lymphocytes, and about 60–80% of peripheral blood T lymphocytes are CD4 cells (T_4). CD4 cells are also the predominant T lymphocytes found in the lymph nodes. CD8 cells (T_8) make up only 35% of peripheral blood T lymphocytes but are the predominant T lymphocyte found in the bone marrow. The normal T_4-to-T_8 ratio in circulating blood is thus ~2:1. The balance between T_4 and T_8 is critical for normal activity of the immune system. This ratio can be decreased in viral infections, immune deficiency states, and acquired immune deficiency syndrome (AIDS) and can be increased in disorders such as acute graft versus host disease, scleroderma, and multiple sclerosis.

γδT Lymphocytes

In the fetal thymus, the first TCR gene rearrangement involves the γ and δ loci. Only ~10% of double negative thymocytes mature to γδTCR T cells after birth.

Natural Killer Cells

The third population of lymphoid cells, **natural killer (NK) cells**, are actually effector cells of innate immunity, not adaptive immunity. Their CD markers are not characteristic of either T or B cells, and they do not rearrange either the BCR or TCR gene loci. However, they do have a diversity of receptors that allow them to recognize appropriate target cells and destroy them. NK cells possess CD56 and CD16 (the $F_c\gamma$ receptor III for IgG). The CD16 marker is also found on neutrophils and some macrophages. NK cells express some T-cell markers on their cell membranes (up to 50% of NK cells can have weak expression of CD8 and CD2), but they lack CD3, CD4, and the TCR.[14]

NK cells originate in the bone marrow from the CLP (Figure 8-8 ■). NK cells and T cells have a close developmental relationship with evidence that the immediate precursor of an NK-committed progenitor is a bipotential CFU-T/NK.[12] IL-15, Flt-3 ligand, SCF, IL7, and IL3 are the major cytokines regulating NK cell differentiation and development (Figure 8-8). NK cells constitute 5–15% of the circulating lymphocytes in the blood and spleen. Most NK cells are short lived with life spans from a few days to a few weeks.[14]

Natural Killer T (NKT) Cells

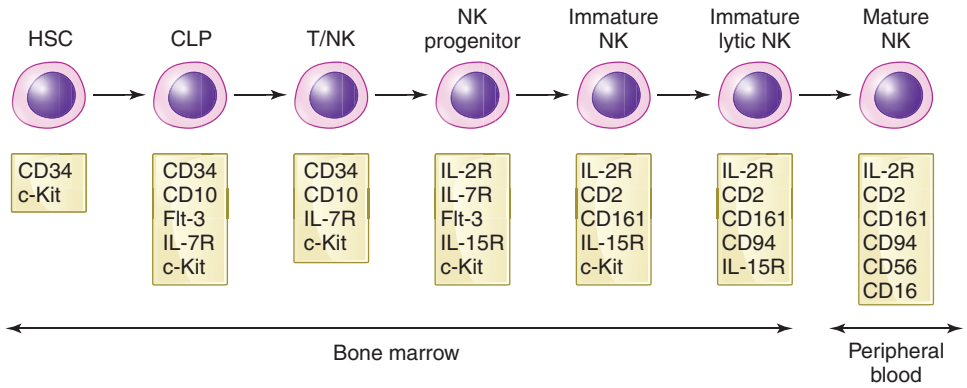
Natural killer T (NKT) cells possess characteristics of both NK and T cells. Their cytotoxic activities are not MHC-restricted, and they do not recognize peptides presented by antigen-presenting cells, both characteristics of cytotoxic T cells. However, they express αβTCRs and carry NK surface markers.[2,15]

CASE STUDY *(continued from page 123)*

1. What class of lymphocytes would you expect to make up the majority of peripheral blood lymphocytes in this child and which subclass CD marker is present on the majority of these cells?
2. Where does this class of lymphocytes differentiate?

LYMPHOCYTE IDENTIFICATION AND MORPHOLOGY

Morphologic criteria cannot be used to differentiate between T, B, and NK lymphocytes. When there is a need to distinguish between them, monoclonal antibodies and flow cytometry for specific CD molecules are most often used. Other phenotypic features of lymphocytes that allow them to be identified as T lymphocytes or B lymphocytes include cytochemical staining properties and gene rearrangement of the TCR and BCR gene loci. T lymphoblasts contain nonspecific esterase, β-glucuronidase, and N-acetyl β-glucosamidase and a punctuate pattern of acid phosphatase positivity. The esterase and acid phosphatase stains for B lymphoblasts are either negative or have a scattered granular pattern of positivity.



■ **FIGURE 8-8** Natural killer (NK) cell maturation pathway. Immunologic maturation of NK cells from the pluripotential hematopoietic stem cell (HSC) and common lymphoid progenitor cell (CLP) to the mature NK cell. Each stage of maturation can be defined by specific antigens (CD) and/or the presence of cytokine receptors that appear on the developing cell. NK progenitors and immature cells are found in the bone marrow, and mature NK cells are found in the peripheral blood.

CD = cluster of differentiation; IL-7R = IL-7 receptor; c-Kit = stem cell factor receptor, also referred to as CD117

☑ CHECKPOINT 8-4

CD markers identify lymphocytes. What are the CD markers for B-cell precursors and T-cell precursors?

Morphology of Immature Lymphocytes

Three *morphologic* stages of lymphoid maturation are recognized: lymphoblast, prolymphocyte, and lymphocyte. The morphologic changes that occur during differentiation or activation are shared by the major lymphocyte subgroups; thus T, B, and NK lymphocytes and their precursors are indistinguishable by morphologic criteria.

Lymphoblast

The lymphoblast (Table 8-2 ★) is about 10–18 mcM (μm) in diameter with a high N:C ratio. The nuclear chromatin is lacy and fine but appears more smudged or heavier than that of myeloblasts. One or two well-defined pale blue nucleoli are visible. The nuclear membrane is dense, and a perinuclear clear zone may be seen; it has less basophilic agranular cytoplasm than other white cell blasts. Whereas subtle morphologic differences exist, lymphoblasts are usually morphologically indistinguishable from myeloblasts. Cytochemical stains can be used to help identify their lymphoid origin (Chapter 23). Unlike myeloblasts, lymphoblasts stain negative for peroxidase, lipid, and esterase but contain acid phosphatase and sometimes deposits of glycogen (PAS+). Both T and B lymphoblasts contain the DNA polymerase TdT.

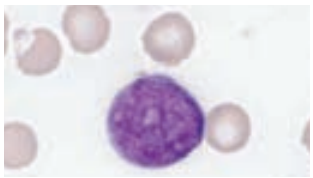
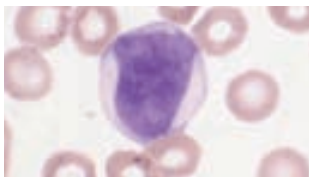
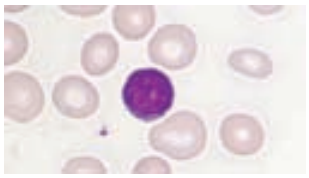
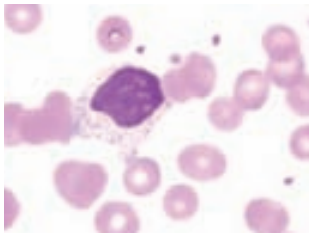
Prolymphocyte

The prolymphocyte is difficult to distinguish in normal bone marrow specimens (Table 8-2). The prolymphocyte is slightly smaller than the lymphoblast with a lower N:C ratio. The nuclear chromatin is clumped but more finely dispersed than that of the lymphocyte. Nucleoli are usually present, and the cytoplasm is light blue and agranular.

Lymphocyte

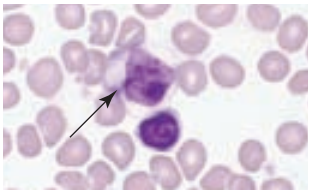
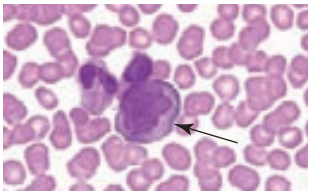
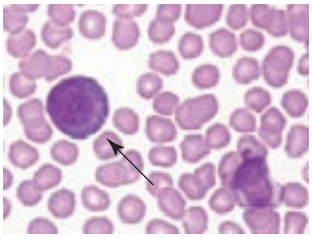
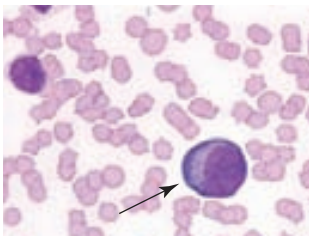
The mature lymphocyte has wide size variability, 7–16 mcM when flattened on a glass slide. Size primarily depends on the amount of cytoplasm present (Table 8-2). Small lymphocytes range in size from

★ **TABLE 8-2** Lymphocyte Maturation

Cell Name	Shape/Size/N:C Ratio	Nucleus	Cytoplasm
Lymphoblasts 	**Shape** Round to oval **Size** 10–18 mcM **N:C Ratio** 6:1–4:1	**Shape** Round to ovoid, centrally located; can be slightly indented, clefted, or folded **Nucleoli** One or more can be present **Chromatin** Lacey or coarsely granular with distinct parachromatin	Scant amount, usually agranular Occasional azurophilic granules and vacuoles
Prolymphocyte 	**Shape** Round to oval **Size** 12–20 mcM **N:C Ratio** 5:1–3:1	**Shape** Round to ovoid, centrally located **Nucleoli** Single prominent **Chromatin** Condensed with indistinct parachromatin	Moderate amount of cytoplasm, occasional azurophilic granules
Small lymphocyte 	**Shape** Round to oval **Size** 7–10 mcM **N:C Ratio** 5:1–2:1	**Shape** Round to ovoid, slightly indented or notched **Nucleoli** Generally not visible or can be inconspicuous **Chromatin** Diffusely dense; blocks of heterochromatin	Scant amount of blue, agranular cytoplasm
Large lymphocyte 	**Shape** Round to oval **Size** 11–16 mcM **N:C Ratio** 4:1–2:1	**Shape** Round to ovoid, slightly indented or notched **Nucleoli** Generally not visible, can be inconspicuous **Chromatin** Diffusely dense; blocks of heterochromatin	Increased amount of blue cytoplasm with variable number of pink, coarse, azurophilic granules

(*Continued*)

★ **TABLE 8-2** *Continued*

Cell Name	Shape/Size/N:C Ratio	Nucleus	Cytoplasm
Reactive lymphocyte 	**Shape** Large and irregular **Size** 16–30 mcM **N:C Ratio** 3:1–2:1	**Shape** Round, ovoid, notched, or indented **Nucleoli** Can be visible **Chromatin** Variable Dense as in a mature lymph but also can appear immature as in a blast	Variable amount, may be dark blue; periphery may be light blue; occasional vacuole; azurophilic granules can be present
Immunoblast 	**Shape** Round to oval **Size** 12–25 mcM **N:C Ratio** 4:1–3:1	**Shape** Large, round, central **Nucleoli** Prominent **Chromatin** Fine	Moderate amount; deep blue
Plasmacytoid lymphocyte 	**Shape** Round to oval **Size** 15–20 mcM **N:C Ratio** 4:1–3:1	**Shape** Round, central, or slightly eccentric **Nucleoli** Single visible **Chromatin** Less clumped and coarse than in plasma cell	Moderate amount, deeply basophilic
Plasma cell 	**Shape** Round to oval **Size** 14–20 mcM	**Shape** Round, eccentrically placed **Nucleoli** Not present **Chromatin** Coarse, blocklike radial masses	Moderate to abundant, deeply basophilic; paranuclear Golgi complex stains poorly and appears as a lighter area

7–10 mcM. The nucleus is about the size of an erythrocyte and occupies about 90% of the cell area; the chromatin is deeply condensed, staining a dark purple. Nucleoli, although always present, are rarely visible with the light microscope. If seen, they appear as small, light areas within the nucleus. A narrow rim of sky blue cytoplasm surrounds the nucleus. A few azurophilic granules and vacuoles can be present. Lymphocytes are motile and can show a peculiar hand mirror shape on stained blood smears with the nucleus in the rounded anterior portion (*protopod*) trailed by an elongated section of cytoplasm known as the *uropod*. Cells that are morphologically small lymphocytes represent a variety of functional subsets of lymphocytes including immunocompetent naïve cells, differentiated effector cells, and memory T and B lymphocytes. Functionally, small lymphocytes are "resting" cells in G_0 of the cell cycle (i.e., they are not actively dividing). However, in response to binding antigen, these small lymphocytes can enter G_1 and initiate the proliferative cell cycle.

Large lymphocytes are heterogeneous in appearance and range in size from 11–16 mcM in diameter. The nucleus can be slightly larger than in the small lymphocyte, but the difference in cell size is mainly attributable to a larger amount of cytoplasm. The cytoplasm can be lighter blue with peripheral basophilia or darker than the cytoplasm of small lymphocytes. Azurophilic granules can be present; if prominent, the cell is described as a large granular lymphocyte. These granules differ from those of the myelocytic cells in that they are peroxidase negative. The nuclear chromatin can appear similar to that of the small lymphocyte or more dispersed, and the nucleus can be slightly indented. Like small lymphocytes, these large cells probably represent a diversity of functional subsets. Large lymphocytes are T or B cells that have encountered antigen and have moved out of G_0.

Normally ~3% of blood lymphocytes are **large granular lymphocytes (LGLs)**. These cells consist of a mixed population of

both NK cells (CD3−) and some activated cytotoxic T cells (CTC, CD3+, CD8+).[16] Most (but not all) NK cells have an LGL morphology, but not all LGLs are NK cells.[9,16] LGLs have a round or indented nucleus and abundant pale blue cytoplasm containing coarse pink granules (Table 8-2). The granules, thought to be related to the cells' cytolytic capacity, contain the pore-forming proteolytic enzyme perforin and granzymes (serine proteases with pro-apoptotic activity).

Morphology of Activated Lymphocytes

During their development into immunocompetent T and B lymphocytes, the cells acquire specific TCRs and BCRs receptors that endow them with antigen specificity. During the antigen-dependent phase of lymphocyte development, contact and binding of this specific antigen to receptors on immunocompetent T and B lymphocytes begin a complex sequence of cellular events known as **blast transformation** (*blastogenesis*). The end result is the clonal amplification of cells responsible for the expression of immunity to that specific antigen. Usually occurring within the lymph node, this series of events includes cell enlargement, an increase in the rough endoplasmic reticulum (RER), enlargement of the nucleolus, dispersal of chromatin, and an increase in DNA synthesis and mitosis. These transformed cells, called **immunoblasts**, have the option to differentiate into terminally differentiated effector cells capable of mediating the immune response or long-lived memory cells. The morphologically identifiable forms of antigen-stimulated lymphocytes include the reactive lymphocyte, reactive immunoblast, plasmacytoid lymphocyte, and plasma cell. Reactive lymphocytes and immunoblasts can be either T or B lymphocytes, which can be determined only by cell marker studies (flow cytometry).

Reactive Lymphocyte

The **reactive lymphocyte** (Table 8-2) can exhibit a variety of morphologic features, including one or more of the following:

- It is increased in size (16–30 mcM) with a decreased N:C ratio.
- The nucleus can be round but is more frequently elongated, stretched, or irregular.
- The chromatin becomes more dispersed, staining lighter than the chromatin of a resting lymphocyte, and nucleoli can be seen.
- There usually is an increase in diffuse or localized basophilia of the cytoplasm, and azurophilic granules and/or vacuoles can be present.
- The cytoplasmic membrane can be indented by surrounding erythrocytes, which gives the cell a scalloped edge.

The reactive lymphocyte has also been referred to as a *stimulated*, *transformed*, *atypical*, *activated*, or *variant lymphocyte*. However, the word *atypical* carries the connotation of abnormal, and therefore some hematologists prefer not to use the term to describe normal lymphocytes in various stages of antigenic stimulation.

A few reactive lymphocytes can be seen in the blood of healthy individuals, but they are found in increased numbers during viral infections. For this reason, the reactive lymphocyte has also been called a *virocyte*.

Immunoblast

The immunoblast (Table 8-2) is the next stage in blast transformation. The cell is large, 12–25 mcM in size, and is characterized by prominent nucleoli and a fine nuclear chromatin pattern (but coarser than that of other leukocyte blasts). The large nucleus is usually central and stains a purple-blue color. The abundant cytoplasm stains an intense blue color due to the high density of polyribosomes. These are cells that are preparing for or engaged in mitosis. The immunoblast proliferates, increasing the pool of cells programmed to respond to the initial stimulating antigen. These antigen-specific daughter cells (effector lymphocytes) mature into cells that mediate the effector functions of the immune response.

The daughter cells of B immunoblasts, which mediate humoral immunity, are plasmacytoid lymphocytes and plasma cells (Table 8-2). Humoral immunity is the production of antibodies by activated B lymphocytes stimulated by antigen.

☑ CHECKPOINT 8-5

A young adult patient has a WBC count of 10×10^9/L with 80% lymphocytes. The blood smear reveals 70% reactive lymphocytes and 10% nonreactive lymphocytes. What is the absolute concentration of total lymphocytes and reactive lymphocytes? What is a probable cause of these findings?

Plasmacytoid Lymphocyte

The **plasmacytoid lymphocyte** (also referred to as *lymphocytoid plasma cell*) (Table 8-2) is believed to be an intermediate cell between the B lymphocyte and the plasma cell. It gains its descriptive name from its morphologic similarity to the lymphocyte but has marked cytoplasmic basophilia similar to that of plasma cells. The plasmacytoid lymphocyte ranges in size from 15–20 mcM. The nuclear chromatin is less clumped (more immature) than that of a plasma cell, and it may have a single visible nucleolus. The nucleus is central or slightly eccentric, and the cytoplasm is deeply basophilic. The cell has some cytoplasmic immunoglobulin (cIg) as well as surface membrane immunoglobulin (sIg). This cell is occasionally seen in the peripheral blood of patients with viral infections.

Plasma Cells

Plasma cells (Table 8-2) are round or slightly oval with a 14–20 mcM diameter. The nucleus is eccentrically placed and contains coarse blocklike radial masses of chromatin, often referred to as the *cartwheel* or *spoke wheel arrangement*. Nucleoli are not present. The paranuclear Golgi complex is obvious and surrounded by deeply basophilic cytoplasm. The cytoplasm stains red with pyronine (pyroninophilic) because of the high RNA content. The RER is well developed, and the cytoplasm enlarges because of the production of large amounts of immunoglobulin. Membrane sIg is usually absent, but azurophilic granules and rodlike crystal inclusions of cytoplasmic Ig can be present.

The reactive lymphocyte is commonly found in the blood during viral infection; the immunoblast, plasmacytoid lymphocyte, and plasma cell usually are found only in lymph nodes and other secondary lymphoid tissue. During intense stimulation of the immune system, however, these transformed cells can be found in the peripheral blood due to lymphocyte recirculation.

In contrast to the progeny of the B immunoblast, effector cells produced from the T immunoblast, cytotoxic T cells, or T helper cells are morphologically indistinguishable from the original unstimulated lymphocytes or appear as LGL.

Memory Cells

A number of the T and B immunoblast daughter cells alternatively form T and B memory cells. Memory cells are morphologically similar to the resting small lymphocytes, retain the ability to react with the stimulating antigen, and are capable of eliciting a rapid secondary immune response when challenged again by the same antigen.

☑ CHECKPOINT 8-6

How would you morphologically differentiate a reactive lymphocyte from a plasma cell on a peripheral blood smear?

LYMPHOCYTE DISTRIBUTION, CONCENTRATION, AND KINETICS

Lymphocytes have a peripheral blood concentration in adults ranging from 1.0–4.8 $\times$ 10^9/L. At birth, the mean lymphocyte count is 5.5 $\times$ 10^9/L. This value rises to a mean of 7 $\times$ 10^9/L in the next 6 months and remains at that level until approximately age 4. A gradual decrease in lymphocytes is noted from 4 years of age until reaching adult reference interval values in the second decade of life. **Lymphocytosis**, an increase in lymphocytes, occurs in adults when the absolute number of lymphocytes is >4.8 $\times$ 10^9/L.

Lymphocytopenia, a decrease in lymphocytes, occurs in adults when the absolute number of lymphocytes is <1.0 $\times$ 10^9/L. Lymphocytes are the second most numerous intravascular leukocyte in adults. However, peripheral blood lymphocytes comprise only ~5% of the total body lymphocyte pool; 95% are located in extravascular tissue of the lymph nodes and spleen. Movement of lymphocytes between the intravascular and extravascular compartments is continuous. Lymphocytes from lymph nodes enter the lymphatic channels and gain entry to the blood as the lymph drains into the right lymphatic duct and the thoracic duct. When stimulated by antigen, lymphocytes migrate to specific areas of lymphoid tissue where they undergo proliferation and transformation into effector cells. To reach extravascular tissue, lymphocytes most often transmigrate between the borders of endothelial cells lining the vessels (paracellular transmigration). Less often, lymphocytes move through endothelial cells (transcellular migration) rather than between them.[1]

Lymphocytes leave and re-enter the blood (recirculate) many times during their life spans. About 80% of lymphocytes in the peripheral blood are "long lived" with a life span ranging from a few months to many years. These cells spend most of their lives in a prolonged, intermitotic, G_0 phase. The remaining 20% of the lymphocytes live from a few hours to ~5 days. The majority of these short-lived, rapidly turning over lymphocytes are immunocompetent, naïve B and T lymphocytes.

CASE STUDY (continued from 132)

3. Calculate the absolute concentration of lymphocytes. Is this increased, decreased, or within the reference interval?
4. If this were a 30-year-old male, would this be considered normal or abnormal? Explain.

☑ CHECKPOINT 8-7

Describe the process of lymphocyte recirculation.

LYMPHOCYTE FUNCTION

T lymphocytes, B lymphocytes, and macrophages interact in a series of events (the adaptive immune response) that allows the body to attack and eliminate foreign pathogens. Cytokines play an important role in activation and regulation of the immune response. Cytokines are produced by a variety of cell types including T lymphocytes, macrophages, dendritic cells, NK cells, and B lymphocytes. These cytokines influence the function of all of the cellular components involved in the innate and adaptive immune responses (Table 8-3 ★). Most cytokines work synergistically in inducing their physiologic response (Chapter 4).

B Lymphocytes (Humoral Immunity)

When naïve B cells encounter antigen in the secondary lymphoid tissues, they are induced to proliferate, resulting in clonal expansion producing large numbers of B cells with the same BCR capable of recognizing that particular antigen. This process is followed by differentiation of the antigen-activated B cell into memory B cells and antibody-secreting plasma cells (the antigen-dependent phase of B lymphopoiesis).

Antigen binding to and activation of naïve B cells results in a primary antibody response, whereas antibody activation of memory B cells results in a secondary antibody response. The secondary response develops more rapidly than does the primary and is associated with predominantly IgG antibody production, whereas the primary response is associated with primarily production of IgM antibodies.

B-cell activation and production of antibodies functions primarily to defend against extracellular bacteria, fungi, and intracellular microbes (before they infect cells). The antibodies function to neutralize and eliminate infectious microbes and microbial toxins. Antibodies are generally produced by plasma cells in the lymphoid organs as well as bone marrow and circulate throughout the body. IgG antibodies coat microbes and promote phagocytosis (opsonization) by binding the Ig Fc region to Fcγ receptors (FcγR-I, -II, -III) on phagocytes. IgG, IgM, and IgA can activate complement, which also generates opsonizing molecules capable of promoting phagocytosis by binding to complement receptors (CR1-4). IgE binds antigen and triggers mast cell degranulation, evoking immediate hypersensitivity reactions (Chapter 7).

★ **TABLE 8-3** Soluble Mediators with Effects on Immune System Cells

Cytokine	Action
MIF	Prevents migration of macrophages from site of inflammation
SCF, Flt3 ligand (FL)	Synergizes with other cytokines to promote proliferation and differentiation of early HSC/HPC; early T- and B-cell development
INFγ	Inhibits intracellular viral multiplication; inhibits proliferation of T_H2 cells; activates macrophages
IL-2	Induces proliferation and activation of lymphocytes (T cells, NK cells)
IL-3	Multilineage colony-stimulating factor; acts in synergy with other cytokines to stimulate proliferation of hematopoietic cells; has a negative effect on lymphoid progenitors
IL-4	Stimulates proliferation of B lymphocytes
IL-5	Stimulates proliferation/differentiation of B lymphocytes and eosinophils
IL-6	Stimulates proliferation/differentiation of B lymphocytes
IL-7	Stimulates proliferation/differentiation of both B- and T-cell progenitors; stimulates proliferation, survival of naïve T cells
IL-9	Stimulates proliferation of T_H cells; potentiates antibody production by B cells; activates mast cells
IL-10	Inhibits production of IL2 and IFNγ by T_H1 cells; suppresses macrophage function
IL-12	Stimulates T cells and NK cells; promotes development of T_H1 cells
IL-13	Stimulates B-cell growth and differentiation; inhibits production of proinflammatory cytokines
IL-14	Stimulates proliferation of activated B cells
IL-15	Activates T cells, NK cells
IL-16	Serves as a chemoattractant of CD4+ lymphocytes
IL-17	Induces endothelial cells, epithelial cells, fibroblasts to secrete other cytokines, including IL-6, IL-8, GM-CSF, EPO, IL-1
IFN-α, -ß	Activate macrophages, granulocytes, CTL
MIP-1α	Responsible for chemotaxis, respiratory burst
TNF-α	Inhibits hematopoiesis

HSC = hematopoietic stem cell; HPC = hematopoietic progenitor cell; IFN = interferon; MIF = macrophage migration inhibitory factor; MIP = macrophage inflammatory protein; SCF = stem cell factor; TNF = tumor necrosis factor

Cell Development

Antigen activation of B lymphocytes results in the morphologic transformation from reactive lymphocytes to immunoblasts, to plasmacytoid lymphocytes, and finally to plasma cells in the lymph nodes. Plasma cells are thus the fully activated terminal effector cell of the B lymphocyte lineage. Their primary function is synthesis and secretion of antibody of the same isotype (Ig class) and idiotype (antigen specificity) as the BCR of its precursor B cell. Plasma cells have large quantities of cytoplasmic immunoglobulin (cIg) but contain little or no surface immunoglobulin (sIg). In contrast, the B cells from which plasma cells are derived are sIg^+ but cIg^-.

Plasma cells are not normally present in the peripheral blood or lymph and constitute <4% of the cells in the bone marrow. Most plasma cells are found in the medullary cords of lymph nodes although intense stimulation of the immune system (e.g., in rubeola, infectious mononucleosis, toxoplasmosis, syphilis, tuberculosis, multiple myeloma) can cause them to be found in the peripheral blood.

Alterations in Ig production are classified as *hypogammaglobulinemia*, *polyclonal gammopathy*, and *monoclonal gammopathy*. Each class of Ig has a specific electrical charge that permits migration in an electrical field. Thus, alterations of Ig production can be detected by serum protein electrophoresis. Hypogammaglobulinemia is a decrease in the total concentration of Ig. Polyclonal gammopathies result in an increase in Ig of more than one class (polyclonal antibodies) and are frequently seen in viral or bacterial infections. Monoclonal gammopathies arise from one clone of cells and are characterized by an increase in one specific class of Ig with identical heavy and light chains. This type of alteration is usually the result of unregulated proliferation (neoplastic) of a clone of plasma cells (Chapter 28). A balance between production of heavy and light chains normally ensures no excess of one or the other. Neoplastic diseases of plasma cells, however, can upset the balance; excesses of light chains or heavy chains can sometimes be found in both serum and urine.

☑ CHECKPOINT 8-8

Where are the majority of plasma cells found, and what is their function?

T Lymphocytes (Cell-Mediated Immunity)

T lymphocytes confer protection against *intracellular* pathogens that have the ability to avoid contact with antibodies. In addition to eradicating microbes, T cells secrete cytokines, which activate other cells such as macrophages and B lymphocytes. Unlike B cells, which can recognize and interact with intact pathogens/antigens, T cells recognize only short peptide fragments derived from protein antigens that are bound by MHC molecules on the surface of the individual's own cells. Thus, T lymphocytes are said to be "MHC-restricted" in terms of their immune response. Some T cells must interact with other cells of the immune system, such as macrophages, dendritic cells, and B lymphocytes. Other T cells must be able to interact with any infected host cell. In both instances, the TCR will recognize only antigens presented by self-MHC molecules.

Recognition of and binding to a target cell requires the TCR and the T-cell coreceptor (CD4 or CD8) to bind with the antigen/MHC. MHC Class I molecules (found on all cells of the body) form a complex with peptides derived from intracellular pathogens and present these antigens to CD8+ T cells (i.e., CD8+ cells are MHC Class I restricted). Class II MHC molecules complex with peptides derived from phagocytosed extracellular pathogens and present them to CD4+ T cells (CD4+ cells are MHC Class II restricted).

Antigen-Presenting Cells

The term **antigen-presenting cell (APC)** is used to identify specialized cells that process and display antigens to CD4+ T lymphocytes. The most effective APC for activating naïve T cells are dendritic cells (DC). Macrophages and B cells primarily function as APCs for memory CD4+ cells.[15] The APC also provide costimulatory signals and cytokines required for T-cell differentiation into effector cells. In return, the T cells secrete cytokines (e.g., IFNγ) that activate the APCs. This positive feedback loop maximizes the immune response.[15]

DCs are found in skin, respiratory and gastrointestinal epithelia, and connective tissues, the usual portals of entry to the body for pathogens. DCs arise from bone marrow precursors, and the majority of DC are related to mononuclear phagocytes (conventional DC or myeloid DC). They are distinguished by their extensive membranous spiny projections. A minor subset of DCs is referred to as *plasmacytoid DCs*, which resemble plasma cells morphologically but acquire the morphology and functional properties of DCs when activated.

Other types of cells can function as APC. Macrophages can present antigens derived from phagocytosed microbes to effector T_H cells. B lymphocytes can internalize protein antigens, degrade them, and present peptides to CD4+ T_H cells. All nucleated cells can become infected with intracellular pathogens and can present peptides derived from pathogen antigens to CD8+ cells, activating a cytolytic response (CTL).

CD4+ T cells

The three types of CD4+ effector cells, T_H1, T_H2, and T_H17, produce different cytokines when activated and are effective against different types of infectious pathogens.[17] Different cytokines that are produced by immune cells (APCs, NK cells, mast cells) at the site of the immune response drive the development of these CD4+ subsets. Differentiation of activated CD4+ T cells to T_H1 effectors occurs in response to intracellular bacteria (e.g., *Listeria*, mycobacteria), whereas T_H2 differentiation occurs in response to helminthes and allergens. Activated **T_H17 cells** provide protection against pathogens by attracting neutrophils and other inflammatory cells to the site of infection.[18,19] Each subset of differentiated effector cells produces cytokines that promote its own development and can suppress the development of the other subsets.

A fourth subset of CD4+ T cells are T_{REG}, which are important in suppressing immune responses and maintaining self-tolerance.[20] In the past, lymphocytes capable of regulating the immune response were described as T suppressor cells (Ts) and were thought to be CD8+ cells. More recently, this suppressor function has been recognized as belonging to a unique subset of lymphocytes, the T_{Reg} cells. T_{Reg} cells are generated when developing thymocytes recognize and strongly bind self-antigens in the thymus and when self- and foreign antigens are recognized in peripheral lymphoid organs. See Table 8-4 ★ for a summary of the CD4+ T-cell functions.

CD8+ T Cells

Activation of naïve CD8+ cells and differentiation into CTLs requires both antigen presentation by MHC Class I molecules and secondary signals, sometimes provided by CD4+ T_H cells.[21] As CD8+ T cells differentiate into effector CTLs, they acquire the machinery to perform target cell killing and develop cytoplasmic granules containing perforin and granzymes that function to kill other cells. On contact with their target, perforin released from the granules introduces a hole in the target cell membrane, allowing activated granzymes and other granule contents to enter. Granzymes activate the target cell apoptotic pathway, inducing target cell death. Fas ligand and other apoptosis-inducing cytokines can also contribute to cytotoxicity through activation of Fas death-receptors on target cells (Chapter 2).

Memory T Cells

In addition to generation of various effector cell subsets, T-cell–mediated immune responses generate memory T cells specific for that antigen that can persist for years.[17] Memory cells survive in a quiescent state (G_0) after antigen is eliminated yet can produce a larger and more rapid response to an antigen than naïve cells on re-exposure. The ability to survive for extended periods of time is due to the upregulation of antiapoptotic proteins in memory cells, including Bcl-2 and Bcl-X_L (Chapter 2).

Natural Killer Cells

NK cells are effector cells of innate immunity whose main function is to kill infected cells and to activate macrophages to destroy phagocytosed microbes.[2,22] NK cells are capable of spontaneous (direct) cytotoxicity for various target cells, primarily cells infected with viruses and intracellular microbes and some tumor cells. They have similar effector functions as CD8+ CTLs, but their cytotoxicity is non-MHC restricted (they do not require interaction with self-MHC molecules on the target cells).[22]

NK cells can recognize and attack pathogens with attached IgG via their IgG receptor (CD16) (*antibody-dependent cellular cytotoxicity* [ADCC]). In addition, they express a variety of receptors that recognize cell surface proteins that are altered when a cell is infected

★ **TABLE 8-4** CD4+ T-Cell Functions

Type	Stimulating Cytokine	Effector Cytokines	Functions	Detrimental Effects
T_H1	IL-12 IFNγ	INF_γ IL-2, TNF_α, TNF_β	Targets intracellular pathogens, activates macrophages, promotes production of IgG_2	Autoimmunity, organ transplant rejection, DTH
T_H2	IL-4	IL-4, IL-5, IL-13, TGF_β	Targets extracellular pathogens, activates eosinophils and mast cells, promotes B cell production of IgE	IgE-mediated allergies and asthma
T_H17	IL-1, IL-6	IL-17, IL-21 IL-22	Combats extracellular microbes; recruits neutrophils	Arthritis Encephalomyelitis
T_{Reg}	IL2, TGF_β	IL10, TGF_β	Inhibits immune response; promotes self-tolerance	Systemic inflammatory disease

("activating receptors"). They also contain "inhibitory receptors" which recognize MHC Class I molecules of autologous cells and block NK cell activation, thus protecting healthy self-cells from an NK attack. The activity of NK cells is carefully regulated and is activated by signals from these various receptors, listed in Table 8-5 ★.

Like CTLs, NK cells rely primarily on the perforin–granzyme system and Fas-Fas-ligand to kill their targets.[22] Because NK cytotoxicity is spontaneous (does not require clonal expansion and activation as do CTLs), NK cells can kill viral-infected cells before antigen-specific CTLs are fully active, early in the viral infection.

A number of cytokines, including IL-2, IL-15, IL-12, and IL-18, activate NK cells. These cytokine-activated NK cells are called **lymphokine-activated killer (LAK) cells**. NK cells produce a variety of cytokines, including G-CSF, GM-CSF, IL-5, TNF, IFNγ, and TGF$_\beta$. Thus they are able to stimulate and inhibit hematopoiesis, and activate cells of both the innate and adaptive immune systems[14,23] (Chapter 4).

Adhesion Molecules of the Adaptive Immune Response

Adhesion receptors are important in the migration of lymphocytes between the blood and lymphoid tissues and for the interaction between immune cells during an immune response (Table 8-6 ★). Most adhesion molecules important for adaptive immune system function belong to either the integrin or immunoglobulin-like families of cytoadhesion molecules.

Aging and Lymphocyte Function

It is now fairly well established that immunocompetence declines with age.[24] By middle age, the thymus has atrophied to ~15% of its maximum size. Although the total number of T cells does not decline significantly, the number of naïve T cells does, resulting in an immune system that does not respond as quickly or as efficiently to a new antigenic challenge. As the naïve T-cell population declines, there is a higher proportion of memory T cells, many of which gradually reach replicative senescence (the inability to proliferate) as one ages. As a result, one's ability to fight off infections declines. Other observations include an increase in cancers associated with aging, which may be partly explained by a declining ability to detect and correct defective cells. Also the aging immune system is less able to distinguish self from nonself, which results in a higher frequency of autoantibody production with age.

Lymphocyte Metabolism

Lymphocytes contain all enzymes of the glycolytic and tricarboxylic acid cycle. Glucose enters the cell through facilitated diffusion and is catabolized to produce ATP through oxidative phosphorylation. The ATP is used for recirculation and locomotion as well as replacement of lipids and proteins and the maintenance of ionic equilibrium. The HMP shunt provides only a fraction of the needed energy, but it is important for purine and pyrimidine synthesis required for DNA replication and mitosis as well as reducing capability associated with production of NADPH.

CASE STUDY *(continued from page 136)*

5. Is there a need for concern regarding the infant's results? Explain your answer.

★ **TABLE 8-5** Regulation of NK Activity

Source of Activating Signal	Activating Signal	Mode of Action
Cytokines	IFN$_{\alpha/\beta}$	Use stress molecules that serve to signal NK cells in the presence of viral pathogens
	IL12	Are produced by macrophages for NK cell stimulation
	IL-15	
Fcγ receptor (CD16)	Molecule that binds Fc portion of IgG	Allows NK cells to target cells against which a humoral response has been mounted and lyse the cells by antibody-dependent cellular cytotoxicity (ADCC)
Activating and inhibitory receptors	Variety of receptors that bind ligands on both endogenous and exogenous target cells	Either activate or suppress NK cell cytolytic activity
	Activating	
	KIR: 3DS1, 2DS1, 2DS2	
	CD94/NKG2-B,-C,-D,-E,-F	
	NKRp-30,-44,-46	
	CD2 or DNAM	
	Inhibiting	
	KIR-2DL1, -2DL2, -2DL3	
	-2DL4,-3DL1	
	CD94/NKG2A	

KIR = killer cell immunoglobulin-like receptor

★ **TABLE 8-6** Adhesion Molecules Involved in the Interaction of Lymphocytes with Other Cells in the Immune Response

Adhesion Molecule	CD Designation	Expressed By	Ligand	Function
LFA-1 α_L/β_2	CD11a/CD18	Activated leukocytes	ICAM-1, -2, -3 on EC; APC	Mediates interaction of lymphocytes with other cells and with endothelium; provides co-stimulatory signal for cell activation
$\alpha_d\beta_2$	CD11d/CD18	Activated lymphocytes	VCAM-1, ICAM-3	Promotes adhesion to leukocytes and to EC
CD2 (LFA-2)	CD2	T lymphocytes, monocytes	LFA-3 (CD58) on EC; APC	Promotes adherence to EC and APC; mediates activation of T lymphocytes
CD4	CD4	T_H lymphocytes	Class II MHC	Enhances adhesion of T_H lymphocytes to APC, mediates activation of T_H lymphocytes
CD8	CD8	CTL	Class I MHC	Enhances adhesion and activation of CTL to APC
VLA1, VLA2, VLA3 VLA4, VLA5, VLA6	CD49a, CD49b, CD49c, CD49d, CD49e, CD49f	Lymphocytes (VLA-4 also on monocytes)	VLA4 ligand; VCAM-1 on EC; ECM proteins	Increases adhesion of cells in area of inflammation

APC = antigen-presenting cell; EC = endothelial cell; CD = cluster of differentiation; EM = extracellular matrix; LFA = leukocyte function-related antigen; ICAM = intercellular cytoadhesion molecule; MHC = major histocompatibility complex antigens; VCAM = vascular cell adhesion; VLA = very late appearing antigen

Summary

The three major types of lymphocytes are T, B, and NK cells. The lymphoid precursor cell from the bone marrow matures and acquires cellular and molecular characteristics of a T, B, or NK lymphocyte. T and B lymphocytes have antigen-independent and antigen-dependent phases of maturation. In the antigen-independent maturation process, immunocompetent B lymphocytes develop in the bone marrow and T lymphocytes develop in the thymus. In the antigen-dependent maturation process, these lymphocytes mature into effector T and B lymphocytes. The immunocompetent cells undergo a series of cellular events in response to encounters with antigens known as *blast transformation*. The end result is a clonal amplification of lymphocyte effector cells responsible for immunity to the specific antigen that stimulated transformation as well as the generation of long-lived memory cells. Reactive lymphocytes are the most common morphologically identifiable form of antigen-stimulated lymphocyte found in the peripheral blood.

T and B lymphocytes have separate but related functions in the immune response. Monocytes/macrophages and dendritic cells phagocytize antigens, generating critical antigenic fragments that are presented to T lymphocytes in a complex with MHC molecules. The T lymphocytes bind to the antigens and the MHC molecules on the surface of the macrophage by means of the TCR and either CD4 or CD8. Cytokines released by the macrophage and the T lymphocyte activate each other reciprocally. There are at least three major types of T lymphocytes: CD4+ helper T lymphocytes (T_H), T_{Reg}, and CD8+ CTLs. The B lymphocyte's functional activity includes differentiation into effector plasma cells and the synthesis and secretion of antibodies. Surface immunoglobulin serves as the B lymphocyte receptor.

NK cells appear morphologically as LGLs and are cytotoxic lymphocytes that function in the innate immune response. Cells morphologically identified as LGLs consist of two distinct populations categorized by the presence or absence of CD3. Natural killer cells are CD3− (NK-LGL), and activated CTLs are CD3+ (T-LGL). The cytotoxicity of NK cells is not MHC restricted.

Review Questions

Level I

1. The function of the lymphocyte is: (Objective 1)
   A. phagocytosis
   B. antigen recognition
   C. hypersensitivity
   D. allergic response
2. Which description best fits the plasma cell? (Objectives 5, 6)
   A. high N:C ratio, nuclear chromatin lacy and fine, nucleoli present, agranular cytoplasm
   B. nucleus eccentric, chromatin in a cartwheel arrangement, deeply basophilic cytoplasm
   C. decreased N:C ratio, nucleus irregular, abundant cytoplasm indented by erythrocytes
   D. round nucleus, abundant cytoplasm, cytoplasm contains large pink granules
3. Lymphocytes can be differentiated from monocytes because lymphocytes have: (Objective 5)
   A. cytoplasm with fine granules and a lobulated nucleus
   B. lacey chromatin pattern with a low N:C ratio
   C. low N:C ratio with intense cytoplasmic basophilia
   D. round dense chromatin nuclear pattern and sky blue cytoplasm
4. A cell on a Wright's stain peripheral blood smear was observed with a stretched or irregular nucleus; occasional nucleoli were present. The cytoplasm was abundant and scalloped around the erythrocytes. This description best fits the: (Objective 6)
   A. plasma cell
   B. lymphoblast
   C. reactive lymph
   D. monocyte
5. The first immunoglobulin heavy chain produced in the maturing B lymphocyte is: (Objective 5)
   A. α
   B. β
   C. μ
   D. γ
6. The adult reference interval for peripheral blood lymphocytes is: (Objective 3)
   A. $0.2–0.8 \times 10^9$/L
   B. $1.0–4.8 \times 10^9$/L
   C. $2.0–7.0 \times 10^9$/L
   D. $3.9–10.6 \times 10^9$/L
7. An 80% lymphocyte count with a total WBC count of 4.4×10^9/L on an adult indicates a: (Objectives 2, 3)
   A. relative and absolute decrease in lymphocytes
   B. relative decrease in lymphocytes but an absolute number within the reference interval
   C. relative increase in lymphocytes but an absolute number within the reference interval
   D. relative and absolute increase in lymphocytes
8. Lymphocyte concentrations in the peripheral blood are highest at what age level? (Objectives 2, 3)
   A. 6 months
   B. 5 years
   C. 25 years
   D. 75 years
9. Which of the following is a characteristic of B lymphocytes? (Objective 1)
   A. regulate immune response
   B. synthesize antibody
   C. secrete cytokines
   D. are majority of peripheral blood lymphocytes
10. Which of the following are cells of the innate immune system? (Objective 1)
    A. plasma cells
    B. natural killer cells
    C. B lymphocytes
    D. T lymphocytes

Level II

1. Pre-B cells are characterized by the presence of: (Objective 3)
   A. CD4 antigen
   B. CD8 antigen
   C. CD19
   D. CD3
2. A major function of CD4+ lymphocytes is: (Objective 4)
   A. to produce immunoglobulin
   B. cellular immunity
   C. to phagocytize microbes
   D. nonspecific cytotoxic function
3. Natural killer cells are: (Objective 4)
   A. T lymphocytes
   B. B lymphocytes
   C. plasma cells
   D. non-T, non-B lymphocytes

4. The most likely explanation for a patient who has a WBC count of 16 $\times$ 10^9/L with many reactive lymphocytes and a few immunoblasts present is: (Objective 4)
   A. a heightened immune response
   B. early leukemia or lymphoma
   C. the presence of immunodeficiency
   D. qualitatively abnormal lymphocytes
5. A patient has lymphocytic leukemia with 60% lymphoblasts in the peripheral blood. The best way for the clinical laboraty professional to determine whether these are T or B lymphoblasts is to: (Objective 5)
   A. do a TdT stain on the blood
   B. determine the CD surface markers on the blasts by flow cytometry
   C. do a molecular analysis to find oncogenes
   D. send the peripheral blood specimen to cytogenetics
6. A patient diagnosed with infectious mononucleosis would most likely produce: (Objective 7)
   A. monoclonal antibody
   B. paraprotein
   C. polyclonal antibody
   D. M protein
7. A patient who is immunosuppressed would most likely have: (Objective 4)
   A. an increase in CD4+ lymphocytes
   B. lymphocytosis and eosinophilia
   C. a decrease in CD4+ lymphs
   D. an increase in the T-cell to B-cell ratio
8. Interleukin 4 plays a role in: (Objectives 2, 3)
   A. inducing proliferation and activation of lymphocytes
   B. enhancing T lymphocytes cell survival
   C. stimulating proliferation of B lymphocytes
   D. activating macrophages and granulocytes
9. KIR molecules are found on which type of lymphocyte? (Objective 4)
   A. B lymphocytes
   B. T lymphocytes
   C. T_{Reg} cells
   D. NK cells
10. CD surface markers including CD2, CD3, CD5, and CD7 are associated with which subclass of lymphocytes?: (Objective 5)
   A. B lymphocytes
   B. T lymphocytes
   C. T_{Reg} cells
   D. NK cells

Companion Resources

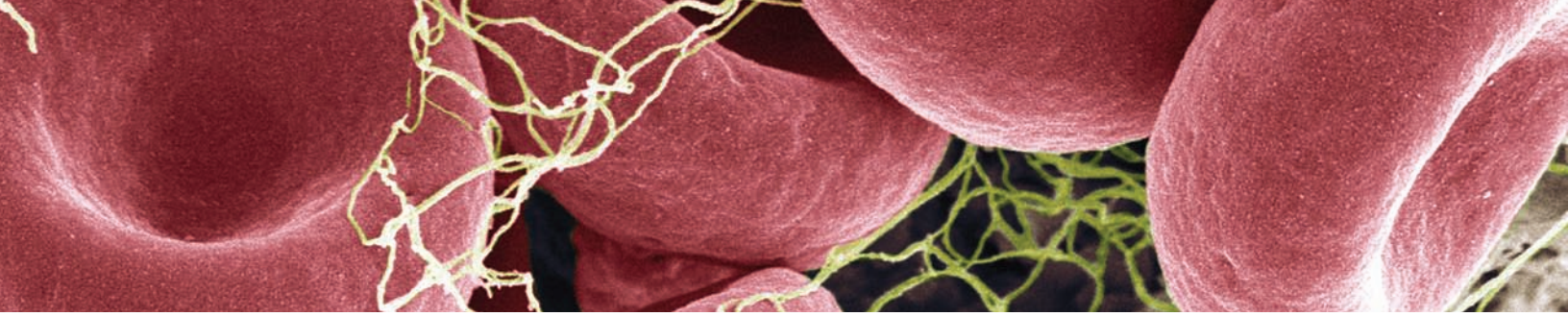
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Abbas AK, Lichtman AH, Pillai S. Leukocyte migration into tissues. In: Abbas AK, Lichtman AH, Pillai S, eds. *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:37–53.
2. Abbas AK, Lichtman AH, Pillai S. Lymphocyte development and antigen receptor gene rearrangement. In: Abbas AK, Lichtman AH, Pillai S, eds. *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:173–202.
3. Parham P. Elements of the immune system and their roles in defense. In: *The Immune System*, 3rd ed. London: Garland Science; 2009:1–28.
4. Shaheen M, Broxmeyer HE. The humoral regulation of hematopoiesis. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Churchill Livingstone; 2009:253–75.
5. Dorshkind K, Rawlings DJ. B-cell development. In: Hoffman R, Benz EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Churchill Livingstone; 2009:105–16.
6. Parham, P. The development of B lymphocytes. In: *The Immune System*, 3rd ed. London: Garland Science; 2009:156–85.
7. Parham, P. The development of T lymphocytes. In: *The Immune System*, 3rd ed. London: Garland Science; 2009:187–208.
8. Douek DC, McFarland RD, Keiser PH et al. Changes in thymic function with age and during treatment of HIV infection. *Nature*. 1998;396:690–95.
9. Powell LD, Baum LG. Overview and compartmentalization of the immune system. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:94–104.
10. McCoy KL. Programmed B and T cell development. *Nutr Rev*. 1998;56(1 Pt 2):S19–S26.
11. Kipps TJ. Functions of T lymphocytes: T-cell receptors for antigen. In: Lightman MA, Beutler E, Kipps TJ et al., eds. *Williams Hematology*, 7th ed. New York: McGraw-Hill; 2006;1065–75.
12. Sanchez M-J, Muench MO, Roncarolo MG et al. Identification of a common T/NK cell progenitor in human fetal thymus. *J Exp Med*. 1994;180:569–76.
13. Spits H. Development of alphabeta T cells in the human thymus. *Nat Rev Immunol*. 2002;2:760–72.
14. Trinchieri G, Lanier LL. Functions of natural killer cells. In: Lightman MA, Beutler E, Kipps TJ et al., eds. *Williams Hematology*, 7th ed. New York: McGraw-Hill; 2006; 1077–82.
15. Abbas AK, Lichtman AH, Pillai S. Major histocompatibility complex molecules and antigen presentation to T lymphocytes. In: Abbas AK, Lichtman AH, Pillai S, eds. *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:109–38.

16. Paraskevas F. T lymphocytes and natural killer cells. In: Greer JP, Foerster J, Lukens J et al, eds. *Wintrobe's Clinical Hematology*, 11th ed. Philadelphia: Lippincott Williams & Wilkins; 2004:475–526.
17. Abbas AK, Lichtman AH, Pillai S. Activation of T lymphocytes. In: Abbas AK, Lichtman AH, Pillai S, eds. *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:203–24.
18. Koenders MI, van den Berg WB. Are T helper 17 cells really pathogenic autoimmunity? *Clin Exp Immunol*. 2009;159:131–36.
19. Lohr J, Knoechel B, Wang JJ et al. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *JEM*. 2006;203:2785–89.
20. Abbas AK, Lichtman AH, Pillai S, eds. Immunologic tolerance and autoimmunity. In: Abbas AK, Lichtman AH, Pillai S. *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:319–44.
21. Abbas AK, Lichtman AH, Pillai S. Effector mechanisms of cell-mediated immunity. In: Abbas AK, Lichtman AH, Pillai S, eds. *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:225–42.
22. Shresta S, Pham CT, Thomas DA et al. How do cytotoxic lymphocytes kill their targets? *Curr Opin Immunol*. 1998;10:581–87.
23. Abbas AK, Lichtman AH, Pillai S. Innate immunity. In: Abbas AK, Lichtman AH, Pillai S, eds. *Cellular and Molecular Immunology*, 7th ed. Philadelphia: Elsevier Saunders; 2012:55–88.
24. Ershler WB, Longo DL. Hematology in older persons. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010. www.accessmedicine.com/content.aspx?aID=6117071 (accessed November 19, 2012).



9 The Platelet

J. LYNNE WILLIAMS, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe the normal morphology and number of platelets on a peripheral blood smear and state the normal life span and concentration of platelets in the blood.
2. Name and describe the cell that is the precursor of platelets in the bone marrow.
3. Define *endomitosis* and *polyploidy*.
4. Identify the major cytokines regulating platelet production.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Define *MPV* and *PDW* and explain their significance in the clinical evaluation of patients.
2. Describe the development of megakaryocytes in the bone marrow to include the stem cell and progenitor cell compartment and the recognizable morphologic stages.
3. Define *proplatelet* and describe the mechanism of platelet release from the marrow to peripheral blood.
4. Explain the significance of megathrombocytes (giant platelets) and reticulated platelets.
5. Describe the effect of thrombopoietin (TPO) on megakaryocytic cells.

Chapter Outline

Key Terms

α-granule (αG)
Demarcation membrane system (DMS)
Dense granule (DG)
Dense tubular system (DTS)
Endomitosis
Mean platelet volume (MPV)
Megakaryoblast
Megakaryocyte
Platelet distribution width (PDW)
Polyploid
Proplatelet
Reticulated platelet
Thrombopoietin (TPO)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the bone marrow production of blood cells. (Chapters 3, 4)

Level II

- Summarize the hierarchy of stem and progenitor cells in the bone marrow and the growth factors that direct the proliferation and maturation of blood cells. (Chapter 4)

CASE STUDY

We will refer to this case study throughout the chapter.

A 70-year-old female saw her physician for symptoms of dizziness and headache. She also had tingling in the hands and feet. Physical exam revealed an enlarged spleen. A CBC was ordered. The hemoglobin was 11.5 g/dL, WBC count was 10×10^9/L, and platelet count was 950×10^9/L. Compare these results to reference intervals.

OVERVIEW

This chapter is an introduction to platelets and provides information essential for the performance and interpretation of a complete blood count (CBC). It describes the evaluation of peripheral blood platelets, including normal physiologic and pathologic alterations that can be seen. In addition, the chapter details the formation of platelets from bone marrow megakaryocytes and their release from the marrow to the peripheral blood. The regulation of megakaryocyte and platelet production, including the effects of cytokines and the marrow microenvironment, is included. A complete description of platelet function is detailed in the section "Hemostasis" (Chapter 31).

INTRODUCTION

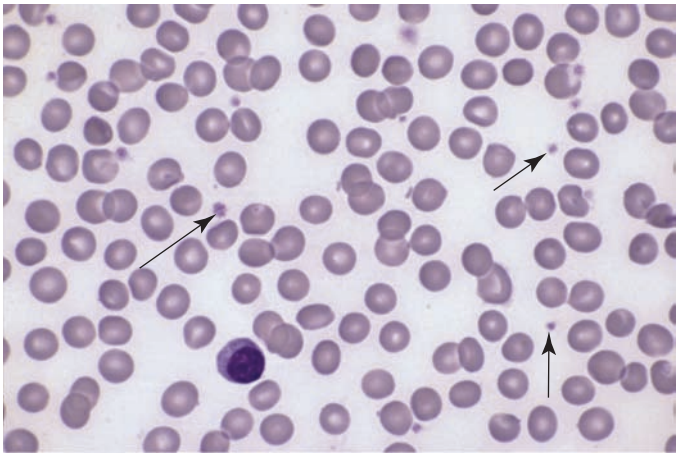
Platelets were once simply considered "blood dust."[1] Studies over the past 150 years have now shown them to play an essential role in hemostasis (the physiology of maintaining blood as a fluid within the circulatory system, and the capacity of blood to clot in the event of vascular injury; Chapter 31). In addition, platelets play an essential role in maintaining vascular endothelial cell integrity and wound healing. Disorders associated with low circulating blood platelets (thrombocytopenia) can be associated with significant morbidity and mortality. Platelets are derived from precursor cells, megakaryocytes, in the bone marrow through a complex biologic process unique to this hematopoietic lineage.

PERIPHERAL BLOOD PLATELETS

Platelets are the smallest of the circulating hematologic elements. They are not truly "cells" but are membrane-bound anucleate fragments of cytoplasm derived from precursor cells in the bone marrow called **megakaryocytes**. Platelets circulate in the peripheral blood for 7–10 days; nonviable or aged platelets are removed by the spleen and liver.

Platelet Morphology

On a Romanowsky-stained peripheral blood smear, platelets appear as small, lavender-blue or colorless bodies with reddish-purple (azurophilic) granules (Figure 9-1 ■). They are generally 2–3 mcM (μm) in diameter, ~0.5 mcM thick, and round to oval in shape. Ultrastructurally, mature platelets lack nuclear material and contain only remnants of a Golgi complex, a relatively small number of ribosomes, and a small amount of mRNA.[2] Newly released platelets, however, contain measurable RNA as do newly released erythrocytes (reticulocytes) (Chapter 5). These immature platelets containing RNA are called **reticulated platelets**, and can be quantitated by some automated hematology instruments using RNA-binding fluorescent dyes. Normal healthy individuals have 5–10% reticulated platelets.[3] It has been proposed that quantitation of reticulated platelets can be used to evaluate marrow megakaryocytopoiesis and to differentiate causes of thrombocytopenia. Thrombocytopenias due to increased destruction of platelets are associated with an increase in reticulated platelets, whereas those due to decreased production have reticulated platelet percentages within the reference interval.[2] An increase in reticulated platelets also correlates with platelet recovery after chemotherapy.[4,5]



■ **FIGURE 9-1** Peripheral blood smear. The arrows point to platelets (Wright-Giemsa stain; 1000× magnification).

Occasionally, a platelet overlies an erythrocyte on a peripheral blood smear and can be mistaken for an erythrocyte inclusion. Differentiation is usually relatively easy because the superimposed platelet has a halo surrounding it and displays the classic morphologic characteristics of a normal platelet (e.g., azurophilic granules). In improperly prepared smears, platelets can appear in aggregates in some areas and appear decreased or absent in others. These aggregates of platelets are typically seen in the "feathered edge" of the blood smear. Aggregated platelets can be seen when the blood is not mixed well with the anticoagulant after collecting the sample, and platelet activation is initiated in vitro. Platelets can also appear decreased due to *platelet satellitosis* (platelet satellitism) when they adhere to neutrophils (Chapter 10). Unusually large platelets—megathrombocytes or *giant platelets* (>5 mcM diameter)—are sometimes seen, particularly in myeloproliferative disorders (Chapter 24) or during recovery from severe thrombocytopenias.

Quantitative Platelet Evaluation

Platelets circulate at a concentration of 150–400 $\times$ 10^9/L. This number is an under-representation of the total number of platelets because normally only two-thirds of peripheral blood platelets circulate and one-third is sequestered in the spleen. These two pools of platelets are in constant equilibrium. In the presence of hypersplenism or splenomegaly, the percentage of sequestered platelets can increase substantially, causing peripheral blood thrombocytopenia. Platelet counts tend to be higher in women than men and decline in both sexes after age 60.[6]

The platelet count was more difficult to automate than either erythrocyte or leukocyte counts because of their small size (potentially difficult to differentiate from cellular debris) and their tendency to aggregate (size of aggregated platelets can overlap size parameter for erythrocytes).[7] However, automated platelet counting by current instrumentation is considered accurate and more precise than manual methods.[8]

A rough estimate of a platelet count can be made by evaluating a peripheral blood smear. With a normal platelet count, there should be 8–20 platelets per oil-immersion field (×1000), or one platelet present for every ~20 erythrocytes.

A useful parameter available with many of the current automated hematology instruments is the **mean platelet volume (MPV)** whose reference interval is ~8–12 fL. Normally, an inverse correlation exists between platelet count and MPV. As a result, there is a relatively stable platelet mass (platelet count $\times$ MPV) across a wide range of normal platelet counts.[9] Originally MPV was believed to be related to platelet age with younger platelets having a larger MPV. Currently, MPV is thought to be related to thrombopoietic stimuli that affect megakaryocyte ploidy.[10,11] An increased MPV can be seen in clinical conditions associated with an increase in thrombopoiesis secondary to thrombocytopenia and in certain pathologic conditions associated with abnormal platelet production such as myelodysplasia. The MPV is artificially increased in samples in which platelet clumping is occurring.

The variation in platelet size seen in the peripheral blood can be described by the **platelet distribution width (PDW)**, a parameter analogous to the red cell distribution width (RDW) (Chapter 10). It has been suggested that the PDW may be useful for distinguishing between reactive thrombocytosis and thrombocytosis associated with myeloproliferative disorders with the latter having a larger PDW than the former.[12] The reference interval for PDW is ~9–15 fL. Reference intervals for both MPV and PDW vary with the instrumentation and methodology used and the population being evaluated. (The PDW is not a Food and Drug Administration–approved parameter for any of the hematology analyzers at the time of printing this book.)

CASE STUDY *(continued from page 145)*

1. Is the patient's platelet count normal or abnormal?
2. Why do you think the spleen is enlarged?

☑ CHECKPOINT 9-1

A patient receiving chemotherapy has a postchemotherapy platelet count of 75 $\times$ 10^9/L, with 24% reticulated platelets. Should the clinician be concerned about the low platelet count?

MEGAKARYOCYTE BIOLOGY

In healthy adults, the bone marrow produces ~1 $\times$ 10^{11} platelets per day. This baseline platelet production can increase 10- to 20-fold in times of increased demand and even further under the stimulation of exogenous thrombopoiesis-stimulating drugs.[13] Platelet production depends on the proliferation and maturation of precursor megakaryocytes in the marrow, a complex process of megakaryopoiesis (megakaryocyte production) and thrombopoiesis (platelet production). Megakaryocytes are rare cells within the bone marrow and comprise only ~0.1% of the total nucleated cells.

Megakaryopoiesis

Platelets are produced in the bone marrow from the same progenitor cell as the erythroid and myeloid lineages (CMP/CFU-GEMM) (Chapter 4). The bipotential myeloid-erythroid progenitor cell (MEP) ultimately gives rise to precursor cells committed to megakaryocytic development (Figure 9-2 ■). The morphologically identifiable platelet precursor cell is called a *megakaryocyte*. Platelets are fragments of the cytoplasm of mature megakaryocytes. The cells of the megakaryocytic lineage include actively proliferating progenitor cells and postmitotic megakaryocytes undergoing maturational development.

Megakaryocyte Progenitor Cell Compartment

Megakaryocyte progenitor cells are responsible for expanding the megakaryocyte numbers and proliferating in response to several hematopoietic cytokines. They are actively proliferating cells and contain the normal 2N/4N DNA content.

The MEP differentiates into a committed megakaryocyte progenitor cell (Chapter 4). Morphologically, early progenitor cells appear as small indistinguishable lymphoid-like cells. They are identified, however, by their growth characteristics in vitro. Using cell culture techniques to grow human adult bone marrow precursor cells, researchers have defined a hierarchy of progenitor cells that develop through stages identified as *BFU-Mk* and *CFU-Mk*.[1,14] Surface

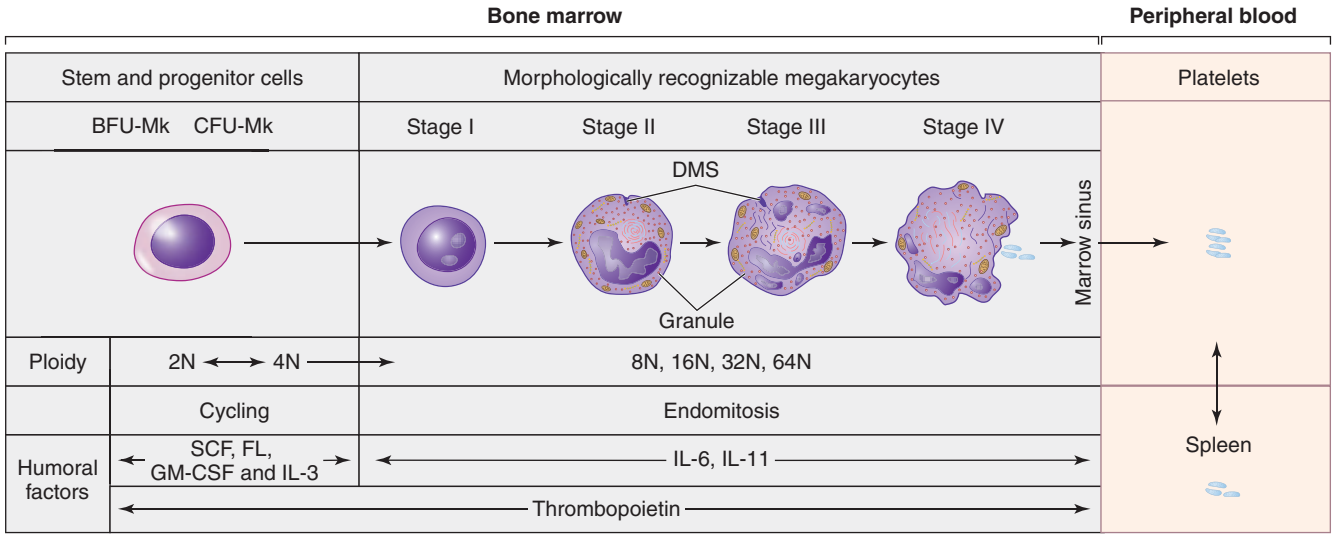


FIGURE 9-2 Megakaryopoiesis. Progenitor cells become committed to the megakaryocyte lineage. Committed progenitor stages are BFU-Mk and CFU-Mk, which can undergo mitosis. Megakaryoblasts stop mitosis and undergo a series of endomitoses until nuclear maturation is complete at 8N to 64N ploidy levels. Cytoplasmic maturation occurs at ploidy levels of 8N or higher. Morphologic features of each stage of cytoplasmic maturation are described in the text. Growth factors Flt ligand, SCF, GM-CSF, and IL-3 influence proliferation of the stem and progenitor cells. Thrombopoietin influences all stages of megakaryocyte production. IL-6 and IL-11 also support megakaryocyte production, primarily maturation.

antigens and growth characteristics differentiate the two stages. The BFU-Mk is $CD34^+$, $HLA\text{-}DR^-$, $c\text{-}Kit^+$ (SCF receptor), whereas the CFU-Mk is positive for all three antigens. In culture, BFU-Mk produce multifocal colonies (bursts) of 100–500 megakaryocytes (6–9 cell divisions) whereas the CFU-Mk produce single colonies of 4–32 megakaryocytes (2–5 cell divisions). A progenitor cell that is more primitive than the BFU-Mk has been grown in cultures of fetal bone marrow called the *high proliferative potential cell-megakaryocyte* (HPPC-Mk).[15] The transit time from megakaryocyte progenitor cell to release of platelets into the circulation is from 4–7 days.[2]

Regulation of Megakaryocyte Production

A regulatory process maintains an adequate number of platelets in the peripheral blood. The bone marrow's production of platelets can increase or decrease according to the body's needs. The major stimulus regulating production is the platelet mass in the circulating blood plus the megakaryocyte mass in the bone marrow.[16]

Humoral Regulation

Several cytokines and growth factors affect megakaryocyte development. Similar to their action on other myeloid cell lineages, interleukin-3 (IL-3), GM-CSF, stem cell factor, and Flt ligand influence the progenitor stages of megakaryocytes to proliferate.[13,14] Interleukin-6 and -11 also affect megakaryocyte development, particularly the maturation phases, but only when they work in synergy and with other cytokines.[13]

The major humoral factor regulating megakaryocyte and platelet development is **thrombopoietin (TPO)**, which influences all stages of megakaryocyte production from the HPPC-Mk level to the release of mature platelets from the bone marrow.[13,16] TPO stimulates megakaryocyte survival and proliferation alone and in combination with other cytokines.[17] TPO also plays an important role in hematopoietic stem cell survival, self-renewal, and expansion (Chapter 4).

Support for TPO as the primary regulator of thrombopoiesis can be seen in humans with congenital amegakaryocytic thrombocytopenia (CAMT) who have inactivating mutations of the thrombopoietin receptor *c-Mpl* (Chapter 33). Children with CAMT present with profound thrombocytopenia and megakaryocytopenia, and most develop aplastic anemia within 5 years of birth.[18,19] Conversely, activating mutations of the TPO or TPO-receptor genes have been identified in cases of familial thrombocytosis.[1] An acquired mutation of proteins involved in the TPO receptor signaling pathway have been found in patients with essential thrombocythemia, a chronic myeloproliferative disorder associated with megakaryocyte hyperproliferation and thrombocytosis (Chapter 24).

The presence of TPO had been suspected since the 1950s, but it was not until 1994 that several research groups first isolated it.[20] The gene for TPO was cloned shortly after its discovery.[21] By 1997, TPO was made by recombinant DNA techniques, and its effects have been studied in vitro and in clinical trials to treat patients with certain platelet disorders.[13] TPO is produced in the liver, kidneys, and spleen and possibly in the bone marrow in patients who have low platelet counts.[22] Thrombopoietin is structurally related to the erythrocyte growth factor, erythropoietin.

In most individuals, plasma TPO levels vary inversely with platelet counts. TPO is thought to maintain a constant baseline number of platelets in the peripheral blood via a unique mechanism called the *sponge model*. Cellular production of TPO is relatively consistent from day to day. TPO binds to its receptor, c-Mpl (CD110), on circulating platelets and bone marrow megakaryocytes and progenitors.[16] TPO bound to circulating platelets is internalized and degraded and

is not available to stimulate proliferation of bone marrow progenitor cells.[23,24] Therefore, the higher the peripheral platelet count, the more TPO is bound with less free TPO remaining in the plasma. This reduces both the stimulation of megakaryocyte progenitor cells in the bone marrow and platelet production. When the platelet count decreases, more TPO is free to bind to megakaryocyte progenitor cells in the bone marrow, increasing platelet production. However TPO synthesis can also be inducible, particularly in times of thrombocytopenia, when TPO mRNA levels can rise.[25]

The effects of TPO in patients with low platelet numbers are to increase the number of megakaryocytes in the bone marrow, the size and DNA content (ploidy level) of megakaryocytes, and the rate of maturation of the megakaryocytes.[13] The number of circulating platelets can increase from 3–10 times the baseline level by administration of TPO.

In addition to the positive regulators of megakaryopoiesis, several substances downregulate megakaryocyte development. Transforming growth factor-β (TGF-β) and α-interferon (IFN-α) both inhibit megakaryopoiesis.[13]

☑ CHECKPOINT 9-2

What would be the effect on the platelet count if a patient had a mutation in the gene for thrombopoietin that resulted in the gene's inability to code for functional mRNA?

Megakaryocyte Microenvironment

Like cells of other hematopoietic lineages, megakaryocytes interact closely with marrow stromal cells. Megakaryocytes are found primarily in the *vascular niche* (Chapters 3 and 4) where they physically attach to endothelial cells lining the sinusoidal vessels. Marrow stromal cells produce both positive and negative regulators of megakaryocyte growth. Stromal cells bear ligands for Notch proteins, which are thought to play a role in influencing the MEP's lineage choice between erythropoiesis and megakaryopoiesis.[26] Stromal cell-derived factor-1 (SDF-1) also contributes to megakaryopoiesis by augmenting TPO-induced megakaryocyte growth and endothelial adhesion within the marrow microenvironment.[27,28]

Stages of Megakaryocyte Development

When TPO and other growth factors stimulate progenitor cell receptors, megakaryocyte differentiation results. The earliest identifiable cell of this lineage is the **megakaryoblast**. Although not easily recognizable morphologically, they can be identified cytochemically by their expression of megakaryocyte-specific markers, such as glycoprotein IIb/IIIa or platelet peroxidase.

The megakaryoblast undergoes a maturation sequence that differs from that of other marrow lineages in that nuclear maturation takes place first and is largely complete before cytoplasmic maturation begins. Following an initial series of proliferative (mitotic) cell divisions during BFU-Mk and CFU-Mk development, the precursor cells begin a unique nuclear maturation process consisting of a series of endomitoses. **Endomitosis** is a unique form of mitosis in which the cell's DNA content doubles, but cell division and nuclear division do not take place.[29] Repetitive cycles result in cells that become **polyploid** with the increased DNA content contained within a single nuclear envelope. Polyploid cells contain exact multiples of the normal DNA content (normally 2N) and can range from 4N to 64N or higher. Endomitosis begins in megakaryoblasts and is completed by the end of stage II megakaryocytes. The 8N stage is generally the first morphologically recognizable stage on a bone marrow smear because by this stage, the megakaryocytes are becoming significantly larger than the other cells in the bone marrow. The 16N stage is the most common ploidy stage in adult humans.

Cytoplasmic maturation can be initiated at nuclear ploidy levels of 8N or more, but the stage at which maturation occurs varies from cell to cell. In general, nuclear maturation (ploidization) ceases when cytoplasmic maturation begins. The reason for variability in cytoplasmic maturation at different ploidy levels is not known. Clinical conditions associated with the presence of large platelets on the peripheral blood smear are noted to have more megakaryocytes with lower ploidy (shifted left). These large platelets are sometimes called *stress platelets* analogous to stress reticulocytes seen in certain anemias. Increased ploidy of an individual megakaryocyte's nucleus generally results in more cytoplasm and, thus, more platelets produced from that megakaryocyte.

Megakaryocyte development occurs as a continuum but is arbitrarily divided into four stages as described on Romanowsky-stained bone marrow smears. The major criteria differentiating these stages include the quantity and characteristics of the cytoplasm and the size, lobulation, and chromatin pattern of the nucleus. The four stages are described in Table 9-1 ★.

Figure 9-3 ■ shows an early (Stage II) megakaryocyte and a mature (Stage IV) megakaryocyte. The nucleus transforms from a single (round) lobe with fine chromatin and visible nucleoli to lobulated with coarse chromatin and no visible nucleoli. In general, as the megakaryocyte matures, the cytoplasm increases in volume and changes from basophilic, nongranular, and scant in the blast stage (Stage I) to completely granular and acidophilic in the mature stage. Developing megakaryocytes produce two types of platelet-specific secretory granules, **α-granules (αGs)** and **dense granules (DGs)**. The cytoplasm in the early stages (Stage I and II) can have a few granules appearing in the region of the Golgi apparatus whereas the cytoplasm of the mature cell (Stage IV) appears completely filled with these azurophilic granules. (The differential morphology and content of these granules is detailed in Chapter 31.)

In addition to granules, the cytoplasm of a maturing megakaryocyte develops an internal membrane system of channels called the **demarcation membrane system (DMS)**. The DMS is not visible by light microscopy but is first seen in electron micrographs at the promegakaryocyte stage. It is derived by invagination of the megakaryocyte's outer membrane and eventually develops into a highly branched, interconnected system of channels that maintain open communication with the extracellular space. Occasionally neutrophils can be seen transiting through the cytoplasm of a mature megakaryocyte via the DMS, a process described as emperipolesis.[13] This observation is not thought to have pathologic significance. Megakaryocytes also produce a second extensive membranous system, the **dense tubular system (DTS)**, which, unlike the DMS, does not communicate

★ **TABLE 9-1** Developmental Stages of Megakaryocytes

	Name	Characteristics
Stage I	Megakaryoblast	6–24 mcM diameter
		Scant basophilic cytoplasm
		No visible granules
		Round nucleus
		Visible nucleoli
Stage II	Promegakaryocyte (basophilic megakaryocyte)	14–30 mcM diameter
		Increased cytoplasm, primarily basophilic
		Few visible azurophilic cytoplasmic granules
		Indented or bilobed nucleus
		Beginning of demarcation membranes (visible with electron microscopy)
Stage III	Granular megakaryocyte	25–50 mcM diameter
		Numerous cytoplasmic granules
		Abundant acidophilic cytoplasm
		Large, multilobed nucleus
		No visible nucleoli
Stage IV	Mature megakaryocyte	40–100 mcM diameter
		Abundant acidophilic, very granular cytoplasm
		Demarcation zones present
		Multilobulated nucleus
		No visible nucleoli

with the cell surface. As the DMS becomes extensive, small areas of the megakaryocyte cytoplasm are compartmentalized (Figure 9-4a ■). These areas eventually become the platelets, and the DMS is thought to provide the material for the formation of proplatelet processes (see "Thrombopoiesis").[30] The separated cytoplasmic areas can be seen on the edges of the cell pictured in Figure 9-3b.

In the practical day-to-day evaluation of bone marrow specimens, distinguishing the maturation stages of megakaryocytes is not necessary. It is, however, important to recognize a cell as being of the megakaryocyte lineage.

☑ CHECKPOINT 9-3

What is the relationship between megakaryocyte ploidy level and eventual number of platelets produced from that megakaryocyte?

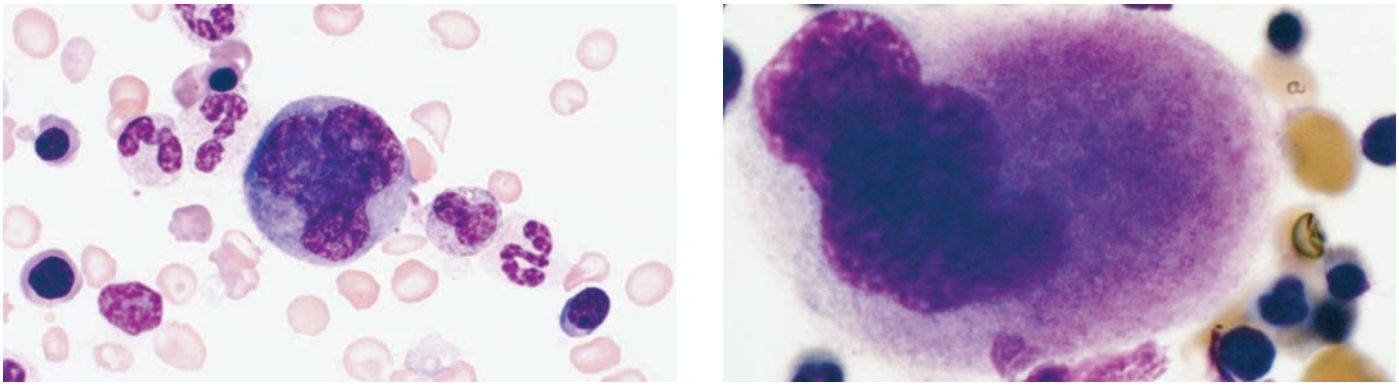
Thrombopoiesis

The primary site of megakaryocyte development (megakaryopoiesis) and platelet production (thrombopoiesis) is in the bone marrow. Mature megakaryocytes are typically situated near the abluminal surface of the marrow sinus endothelial cells and shed platelets directly into the marrow sinuses. Each megakaryocyte is estimated to give rise to 1000–3000 platelets, depending on the ploidy of the parent megakaryocyte.[31] Megakaryocytes of ploidy of 8N or higher can produce platelets (Figure 9-5 ■).

Platelets form by fragmentation of megakaryocyte cytoplasm. They appear to be released from membrane extensions of megakaryocytes in groups called **proplatelets**, which are long slender protrusions of megakaryocyte cytoplasm (Figure 9-4b). Each megakaryocyte extrudes multiple cytoplasmic extensions between endothelial cells into the marrow sinuses as proplatelets, which then break up into individual platelets. The megakaryocyte nucleus remains in the marrow and is engulfed by the marrow macrophages. Localized apoptosis (caspase activation) is thought to play a role in the final stages of platelet formation and release.[32,33] Whole intact megakaryocytes occasionally are released from the marrow, circulate in the peripheral blood, and become trapped in capillary beds in the spleen and lungs. These cells also can release platelets to the peripheral blood, although their contribution to total platelet production is thought to be only 7–15%.[1]

CASE STUDY *(continued from page 146)*

3. A bone marrow examination was ordered. What cells would you expect to see increased? Why? Would this affect the M:E ratio?
4. What cytokine normally stimulates the production of these cells? What is the receptor for this cytokine?
5. The patient was diagnosed with essential thrombocythemia. What is the pathophysiology of this disorder?



a b

■ **FIGURE 9-3** **(a)** Early megakaryocyte. **(b)** Mature megakaryocyte (bone marrow; Wright-Giemsa stain; 1000× magnification).

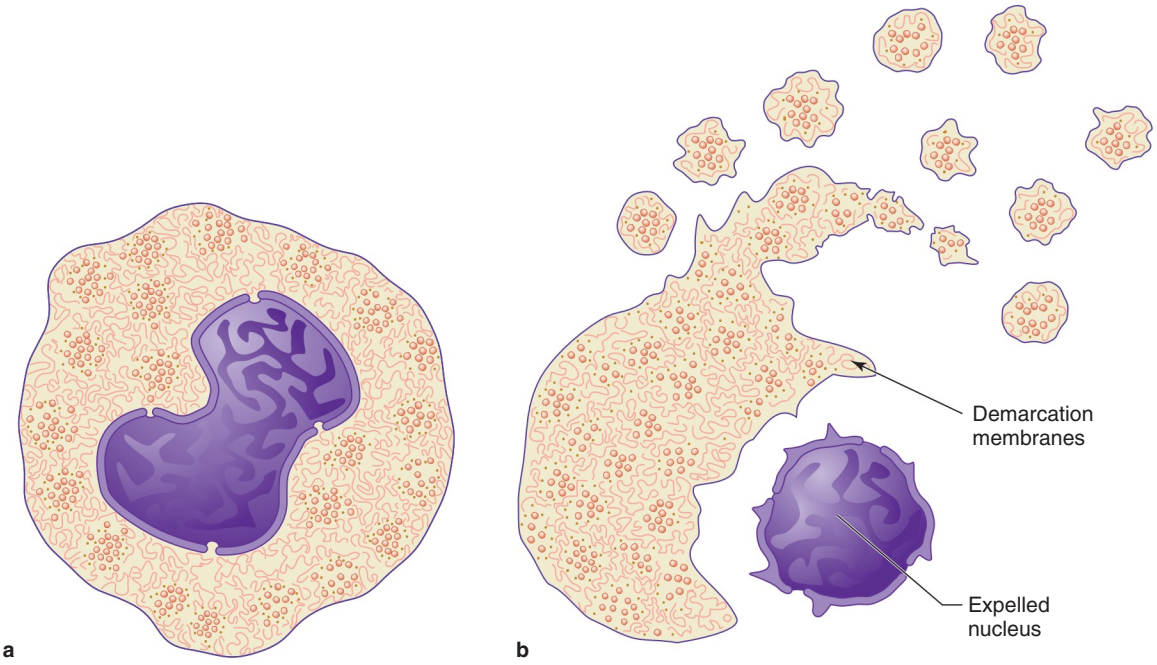


FIGURE 9-4 **(a)** Mature megakaryocyte showing future platelets. **(b)** Platelet release from mature megakaryocyte. Outward extrusion of cytoplasm, eventually breaking up into individual platelets delineated by demarcation membrane system (arrow). Expelled nucleus is phagocytized by marrow macrophages.

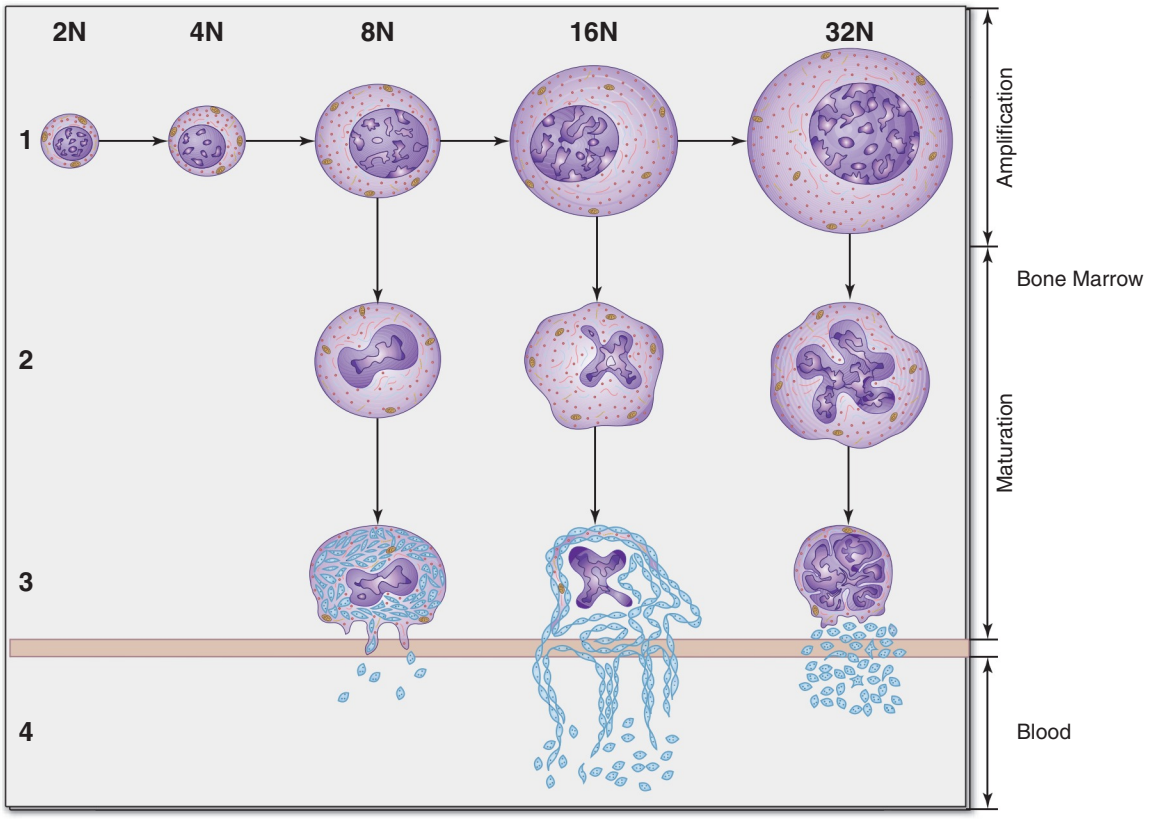


FIGURE 9-5 Platelet production. Developing megakaryocytes generally undergo nuclear maturation (ploidization) prior to cytoplasmic development. Megakaryocytes at any ploidy level 8N or higher can undergo cytoplasmic maturation and platelet production. Platelets are first released between the endothelial cells of the marrow sinuses as proplatelets. proplatelets break into mature platelets and are released into the peripheral blood.

Summary

Peripheral blood platelets are produced by the process of cytoplasmic fragmentation of precursor cells—the megakaryocytes—in the bone marrow. A number of cytokines control megakaryocyte and platelet production; however, thrombopoietin appears to be the major regulator of these processes. A number of marrow microenvironmental factors play a role as well. Megakaryocyte development is characterized by a unique form of mitosis—endomitosis—in which DNA synthesis occurs in the absence of cell or nuclear division, resulting in large, polyploid cells. Four different maturation stages of developing megakaryocytes have been identified, but differentiation and quantitation of these stages is generally not considered clinically significant. On average, 1000–3000 platelets are shed from a single megakaryocyte. One-third of the platelets entering the peripheral blood from the marrow are sequestered in the spleen, and two-thirds freely circulate. Platelet parameters thought to have clinical relevance in evaluating patients include the platelet count, the percentage of reticulated platelets, the mean platelet volume, and the platelet distribution width.

Review Questions

Level I

1. What bone marrow cell is the precursor of platelets? (Objective 2)
   A. myeloblast
   B. erythroblast
   C. endothelial cell
   D. megakaryocyte
2. What is a reasonable reference interval for platelets in the peripheral blood? (Objective 1)
   A. $10–20 \times 10^9$/L
   B. $150–200 \times 10^{12}$/L
   C. $150–400 \times 10^9$/L
   D. $400–600 \times 10^9$/L
3. Which of the following best describes the normal morphology of platelets on a peripheral blood smear? (Objective 1)
   A. They are larger than erythrocytes.
   B. They are filled with azurophilic granules.
   C. They are light blue in color without granules.
   D. They have large nuclei.
4. Which of the following best describe the appearance of mature megakaryocytes in the bone marrow? (Objective 2)
   A. They are large cells with scanty basophilic cytoplasm (high N:C ratio).
   B. They are large cells with multiple discrete nuclei, basophilic cytoplasm.
   C. They have abundant acidophilic cytoplasm filled with azurophilic granules and a single, large, lobulated nucleus.
   D. They are about the size of a marrow macrophage with a high N:C ratio and granular cytoplasm.
5. The major cytokine responsible for regulating both megakaryocyte and platelet production is: (Objective 4)
   A. TPO
   B. IL-11
   C. SCF
   D. IL-6
6. Endomitosis is defined as: (Objective 3)
   A. cell proliferation endogenous to the bone marrow
   B. fragmentation of megakaryocyte cytoplasm to produce platelets
   C. fusion of megakaryocyte progenitor cells to produce polyploidy megakaryocytes
   D. rounds of DNA synthesis without nuclear division or cellular division
7. What is the normal life span of platelets in the peripheral blood? (Objective 1)
   A. 8 hours
   B. 1 day
   C. 10 days
   D. 100 days

Level II

1. Which of the following statements best describes the role of the platelet parameters MPV and PDW in evaluating patients? (Objective 1)
   A. They are theoretical constructs, and we do not yet have the capacity to measure either variable.
   B. They are measurable but not on current laboratory hematology instrumentation.
   C. Variations in both MPV and PDW can be measured, but their clinical significance is unknown.
   D. Variations in both MPV and PDW can be measured and can contribute to the differential diagnosis of platelet disorders.

2. Place the following megakaryocyte precursor cells in the correct developmental sequence: (Objective 2)
   A. BFU-Mk → CFU-Mk → megakaryoblast → granular megakaryocyte
   B. CFU-Mk → BFU-Mk → basophilic megakaryocyte → granular megakaryocyte
   C. BFU-Mk → CFU-Mk → basophilic megakaryocyte → megakaryoblast
   D. CFU-Mk → BFU-Mk → megakaryoblast → basophilic megakaryocyte
3. Which of the following is believed to be the mechanism of platelet formation and release by megakaryocytes? (Objectives 2, 3)
   A. dissolution of the megakaryocyte cell membrane and release of preformed platelets
   B. release of individual reticulated platelets from the cytoplasm
   C. formation of proplatelet processes, which subsequently segment into individual platelets
   D. apoptotic cell death of the megakaryocyte and the disintegration of the cytoplasm as platelets
4. A patient receiving chemotherapy experienced severe marrow suppression and anemia, leukopenia, and thrombopenia as a result. After about one week, the platelet count remained low, and the number of reticulated platelets was above the reference interval for that parameter. This would likely indicate that the: (Objective 4)
   A. patient's marrow is failing to initiate recovery of the thrombocytopenia
   B. patient is a candidate for erythropoietin
   C. patient should receive a blood transfusion
   D. the patient's marrow is showing early signs of recovery of the thrombocytopenia
5. What effect does thrombopoietin have on the megakaryocyte? (Objective 2)
   A. It decreases the ploidy level and number of platelets formed.
   B. It increases size, DNA ploidy level, and rate of maturation.
   C. It induces the release of megakaryocytes to the peripheral blood.
   D. It decreases the rate of endomitosis and increases ploidy level.
6. What is the effect of a significant number of giant platelets (megathrombocytes) resulting in an increased MPV on the peripheral blood platelet count? (Objective 4)
   A. It would have no effect on the platelet count.
   B. It would generally be associated with an increased platelet count.
   C. It would generally be associated with a decreased platelet count.
   D. It would result in an unreliable reading on the automated instrument and require a manual platelet count.

Companion Resources

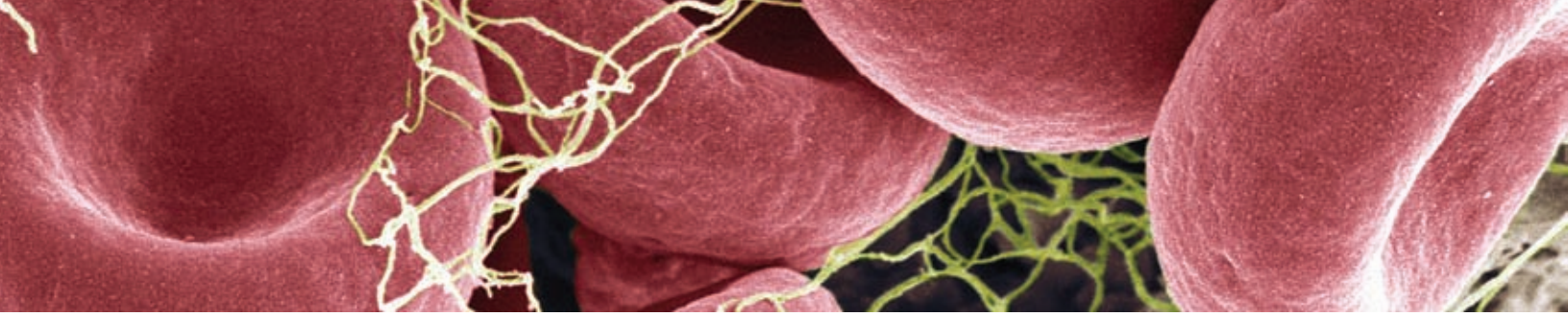
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Cantor AB. Thrombopoiesis. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:305–18.
2. Smyth SS, Whiteheart S, Italiano JE et al. Platelet morphology, biochemistry, and function. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*. 8th ed. New York: McGraw-Hill; 2010. www.accessmedicine.com/content.aspx?aID=6231303 (accessed December 19, 2012).
3. Kurata Y, Hayashi S, Kiyoi T et al. Diagnostic value of tests for reticulated platelets, plasma glycocalicin, and thrombopoietin levels for discriminating between hyperdestructive and hypoplastic thrombocytopenia. *Am J Clin Pathol*. 2001;115:656–64.
4. Chaoui D, Chakroun T, Robert F et al. Reticulated platelets: a reliable measure to reduce prophylactic platelet transfusions after intensive chemotherapy. *Transfusion*. 2005;45:766–72.

5. Wang C, Smith BR, Ault KA et al. Reticulated platelets predict platelet count recovery following chemotherapy. *Transfusion*. 2002; 42:368–74.
6. Bennett JS, Abrams CS. Differential diagnosis of thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS et al., *Hemostasis and Thrombosis, Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott, Williams & Wilkins; 2013:757–62.
7. Ryan DH. Examination of blood cells. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010. www.accessmedicine.com/content.aspx?aID=6106433 (accessed December 4, 2012.
8. Lawrence JB, Yomtovian RA, Dillman C et al. Reliability of automated platelet counts: comparison with manual method and utility for prediction of clinical bleeding. *Am J Hematol*. 1995;48:244-50.
9. Levin J, Bessman JD. The inverse relation between platelet volume and platelet number. Abnormalities in hematologic disease and evidence that platelet size does not correlate with platelet age. *J Lab Clin Med*. 1983;101:295–307.
10. Thompson CB, Love DG, Quinn PG et al. Platelet size does not correlate with platelet age. *Blood*. 1983;62:487–94.
11. Bessman JD. The relation of megakaryocyte ploidy to platelet volume. *Am J Hematol*.1984;16:161–70.
12. Osselaer JV, Jamart J, Scheiff JM. Platelet distribution width for differential diagnosis of thrombocytosis. *Clin Chem*. 1997;43:1072–76.
13. Kaushansky K. Megakaryopoiesis and thrombopoiesis. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010. www.accessmedicine.com/content.aspx?aID=6230972 (accessed December 4, 2012).
14. Gerwirtz AM. Human megakaryocytopoiesis. *Semin Hematol*. 1986;23:27–42.
15. Bruno E, Hoffman R. Human megakaryocyte progenitor cells. *Semin Hematol*. 1998;35:182–91.
16. Wendling F. Thrombopoietin: its role from early hematopoiesis to platelet production. *Haematologica*. 1999;84:158–66.
17. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy *in vitro*. *Blood*. 1995;85:1719–26.
18. van den Oudenrijn S, Bruin M, Folman CC et al. Mutations in the thrombopoietin receptor, Mpl, in children with congenital amegakaryocytic thrombocytopenia. *Br J Haematol*. 2000;110:441.
19. Ballmaier M, Germeshausen M, Schulze H et al. c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. *Blood*. 2001; 97:139-46.
20. Kaushansky K. Thrombopoietin: the primary regulator of platelet production. *Blood*. 1995;86:419–31.
21. Lok S, Kaushansky K, Holly RD et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature*. 1994; 369:565–68.
22. Sungaran R, Markovic B, Chong BH. Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. *Blood*. 1997;89:101–7.
23. Kuter DJ, Rosenberg RD. The reciprocal relationship of thrombopoietin (c-Mpl ligand) to changes in the platelet mass during busulfan-induced thrombocytopenia in the rabbit. *Blood*. 1995;85:2720–30.
24. Emmons RV, Reid DM, Cohen RL et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. *Blood*. 1996; 87:4068–71.
25. McCarty JM, Sprugel KH, Fox NE et al. Murine thrombopoietin mRNA levels are modulated by platelet count. *Blood*. 1995;86:3668–75.
26. Lam LT, Ronchini C, Norton J et al. Suppression of erythroid but not megakaryocytic differentiation of human K562 erythroleukemic cells by notch-1. *J Biol Chem*. 2000;275:19676–84.
27. Hodohara K, Fujii N, Yamamoto N et al., Stromal cell-derived factor-1 (SDF-1) acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells (CFU-MK). *Blood*. 2000; 95:769–75.
28. Wang JF, Liu ZY, Groopman JE. The alpha-chemokine receptor CXCR4 is expressed on the megakaryocytic lineage from progenitor to platelets and modulates migration and adhesion. *Blood*. 1998;92:756–64.
29. Cramer EM. Megakaryocyte structure and function. *Curr Opin Hematol*. 1999;6:354–61.
30. Italiano JE Jr, Shivdasani RA. Megakaryocytes and beyond: the birth of platelets. *J Thromb Haemost*. 2003;1:1174–82.
31. Harker LA, Finch CA. Thrombokinetics in man. *J Clin Invest*. 1969;48:963–74
32. Li J, Kuter DJ. The end is just the beginning: megakaryocyte apoptosis and platelet release. *Int J Hematol*. 2001;74:365–74.
33. De Botton S, Sabri S, Daugas E et al. Platelet formation is the consequence of caspase activation within megakaryocytes. *Blood*. 2002;100:1310–17.



10 The Complete Blood Count and Peripheral Blood Smear Evaluation

KRISTIN LANDIS-PIWOWAR, PHD
JOHN LANDIS, MS
KEILA POULSON, BS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. List the assays typically included in the complete blood count (CBC).
2. Describe how to properly identify a patient prior to blood collection.
3. List the pre-examination precautions that must be observed to produce quality results when performing a CBC.
4. List the typical units of measure for reporting the WBC, RBC, Hgb, Hct, and reticulocyte count.
5. Define the terms *MCV*, *MCH*, *MCHC*, and *RDW*.
6. Given the hemoglobin, hematocrit, and RBC count, demonstrate the ability to use the formulas to calculate the RBC indices.
7. Describe the process of evaluating the peripheral blood smear including macroscopically and microscopically using low and high power magnification microscopic settings.
8. Define *poikilocytosis* and *anisocytosis*, and describe and identify specific poikilocytes and anisocytes.
9. Classify erythrocytes morphologically based on erythrocyte indices.
10. Given the relative reticulocyte count and RBC count, calculate the absolute reticulocyte count.
11. Identify erythrocyte inclusions; describe their composition and staining characteristics.
12. Recognize abnormal variation in erythrocyte distribution on stained smears.
13. Correlate polychromatophilia on a blood smear with other laboratory results of erythrocyte production and destruction.
14. Describe the variations in the CBC reference intervals found in African Americans, newborns, and elderly people.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Interpret RDW results.
2. Assess bone marrow response to anemia given CBC and reticulocyte results.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

3. Identify poikilocytes on a blood smear, describe their mechanism of formation, and correlate them with pathological conditions.
4. Correlate CBC and reticulocyte results with findings on a blood smear and troubleshoot any discrepancies.
5. Select methods for differentiating erythrocyte inclusions.
6. Evaluate distribution of erythrocytes on stained smears, and describe how the distribution could affect the CBC results.
7. Correlate the age of a patient and the typical changes in CBC results compared with a normal adult CBC.

Key Terms

Acanthocyte
Agglutination
Anisocytosis
Basophilic stippling
Cabot ring
Codocyte
Critical area
Critical limit
Dacryocyte/teardrop
Delta check
Drepanocyte
Echinocyte
Elliptocyte
Heinz body
Helmet cell
Hematocrit
Howell-Jolly body
Hypochromic cell
Keratocyte
Knizocyte
Leptocyte
Macrocyte
Mean cell hemoglobin (MCH)
Mean cell hemoglobin concentration (MCHC)
Mean cell volume (MCV)
Microcyte
Normochromic
Normocytic
Ovalocyte
Panic value
Pappenheimer body
Poikilocytosis
Red cell distribution width (RDW)
Reflex testing
Rouleaux
Rule of three
Schistocyte
Sickle cell
Sideroblast
Siderocyte
Spherocyte
Stomatocyte
Target cell
Teardrop/dacryocyte

Background Basics

The information in this chapter builds on the concepts learned in Chapters 4–9. In addition to the basics from previous chapters, it is helpful to have a basic understanding of algebra, the use of percentages, ratios and proportions, statistics, and the metric system. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the basic concepts of cell differentiation and maturation. (Chapter 4)
- Identify the reference intervals for erythrocytes, reticulocytes, and hemoglobin. (Chapters 5, 6)
- Identify the reference intervals for each subclass of leukocytes and for platelets. (Chapters 7–9)

Level II

- Correlate the relationships between various infectious agents and absolute and relative leukocyte counts. (Chapters 7, 8)
- Predict the effects of increased and decreased erythropoietin in the blood. (Chapters 5, 6)
- Summarize the role of thrombopoietin in platelet production. (Chapter 9)

CASE STUDY

We will refer to this case study throughout the chapter.

A 48-year-old woman was admitted to a hospital because of wheezing, cough, fatigue, and difficulty breathing. Her history included diabetes and hypertension. Her CBC data show a hemoglobin of 8.2 g/dL (82 g/L), RBC count of 1.73 × 10^{12}/L, and MCV = 109.8 fL. Her WBC count and platelet count were within the reference interval.

Consider the actions that the clinical laboratory professional should take.

OVERVIEW

This chapter unites the individual classes and characteristics of blood cells that were described in Chapters 4–9 into a clinically relevant "blood picture" of those cells as they circulate in the peripheral blood. A brief discussion of the pre-examination steps that provide the foundation for any routine hematologic evaluation precedes a more in-depth examination of blood cell counts and composition and visual features on a prepared blood smear. Finally, a brief synopsis of the post-examination characterization of the complete blood count will be included. The classification of disease as it is determined from laboratory findings will be detailed in subsequent chapters of this book.

INTRODUCTION

The performance of a complete blood count (CBC) has three phases: pre-examination (before testing), examination (testing), and post-examination (reporting) (Table 10-1 ★). The pre-examination phase includes the proper identification of the patient and proper collection and handling of the specimen. The specimen is analyzed in examination phase. The CBC (sometimes referred to as the *peripheral blood count* [*PBC*]) is a primary screening test that provides information regarding the cellular components of the blood as they circulate in the peripheral blood. The concentration of leukocytes (white blood cell [WBC]), erythrocytes (red blood cell [RBC]), and platelets as well as a categorization of the different WBC subsets is included. Additional information regarding RBCs is also integrated into the CBC and includes, at minimum, the concentration of hemoglobin and the

★ **TABLE 10-1** Phases of the CBC

Pre-examination	Patient identification
	Specimen collection and handling
Examination	Automated results
	Evaluation and analysis of peripheral blood smear
Post-examination	Interpretation of data
	Reporting results

packed cell volume of RBCs, called the hematocrit. Finally, a CBC can also provide what are known as the RBC *indices* that are used to depict the volume and the total weight of each RBC and concentration of hemoglobin in it. The CBC can be determined by automated and/or manual methods, which are described in detail in Chapters 37 and 39. The post-examination phase includes reporting and interpreting the data. Based on the information collected from the CBC, the laboratory professional can provide diagnostic criteria or meaningful recommendations for any follow-up testing (**reflex testing**) to the physician that support quality patient care.

PRE-EXAMINATION PHASE OF THE CBC

The pre-examination phase of the CBC encompasses patient identification, blood collection, and specimen handling (Chapters 37 and 43). Briefly, patient identification includes the patient's name and a second identifier that can be a hospital number but more commonly is the patient's date of birth. This documentation must be available from the patient at the time of the blood collection.

Once the patient has been properly identified, the laboratory professional performing the phlebotomy must be well acquainted with the various collection devices, their safety features and requirements, and the anticoagulants or additives within the sample collection tubes (Chapter 37). This individual should have a thorough knowledge of the phlebotomy procedure safety issues including methods to prevent exposure to blood-borne pathogens.

Although other blood collection tube additives can be used for hematologic analysis, almost all specimens for a routine CBC are collected in a purple-/lavender-top sample collection tube that contains ethylenediaminetetraacetic acid (EDTA). Dipotassium (K_2) EDTA provides the best morphologic preservation of blood cells and prevents coagulation of the blood specimen.[1] The anticoagulated specimen allows a laboratory professional to generate multiple blood smears from one tube of blood, a technique that must be performed within 3 hours of blood collection.[2]

Proper transport is of great concern within a clinical facility and is even more important when samples are brought in from locations outside the facility. Samples from outside facilities must be delivered in a manner that complies with limited temperature variations and time restraints. Freezing or excessive heat will damage the blood cells and render analysis invalid. Although microscopic evaluation of a blood smear is best when the slide is prepared within 3 hours of collection, the instrument analysis can be delayed for 6–8 hours without deterioration of the data.[3] Some parameters are valid for up to 24 hours after sample collection if properly stored. Often, however, the hematology instrument's manufacturer provides the recommended time points for performing the automated CBC to ensure that the analysis produces data for a patient of the highest quality. The information generated from manual and instrument analysis is only as good as the specimen that is tested. Therefore, the pre-examination stage of testing is of primary importance. Sources of error in sample collection and their effect on test outcome is detailed in Chapter 43, Table 43-2.

☑ CHECKPOINT 10-1

A courier service delivers a tube of blood from an outpatient drawing station across town. The requisition for the sample is for a CBC. The blood is in a blue top vacutainer tube and was transported in an insulated box containing dry ice. What are the next steps in processing this sample?

EXAMINATION PHASE OF THE CBC

Automated Results

Laboratory professionals use automated instruments to determine the CBC information for the majority of patient samples (Chapter 39). Proper instrument preparation includes quality control and assessment to confirm the normal function of the instrument. Commercial controls, patient controls, or moving averages are used to determine the analytical reliability of the automated instrument (Chapter 43). Once the analytical reliability of the instrument has been confirmed, examination of patient samples can begin. Throughout the examination phase, laboratory professionals must follow safety protocols designed to minimize risk of exposure to biohazards, chemical hazards, and physical hazards and dispose of biological waste appropriately (Chapter 43).

Each hematology instrument utilizes different but overlapping technologies (e.g., impedance, optical light scattering) to determine the reporting parameters of the CBC. A typical panel of CBC parameters includes information regarding the cells produced by the hematopoietic system, the RBCs, WBCs, and platelets (Table 10-2 ★). Because other organs and organ systems also affect hematopoiesis, a myriad of disease states can be evaluated from CBC data to determine diagnosis, treatment, and prognosis for a patient.

In addition to the typical CBC parameters, hematology instruments generate scatterplots and histograms (refer to Chapter 39 for detailed discussion) that laboratory professionals interpret. Each scatterplot and histogram contains information about the cell populations, interfering substances, and instrument function and therefore serve as forms of quality control for instrument and specimen integrity. For example, the laboratory professional consults the scatterplots and histograms to assess the volume of granulocytes (increased volume indicates immaturity or nuclear hypersegmentation whereas decreased volume of lymphocytes can indicate chronic lymphocytic leukemia). The scatterplots and histograms are also helpful in assessing RBC parameters that can be affected by conditions such as cold **agglutination** (RBC clumping at temperatures below body temperature) and a severely elevated WBC count. Tables 43-8 and 43-9 provide detailed information on abnormal and spurious results, underlying problems, causes, and corrective actions. Hematology reference intervals for the various age groups can be found in the front cover of this textbook.

★ **TABLE 10-2** Parameters and Reference Intervals of a Typical Adult CBC[a]

White blood cell count	$4.5–11.0 \times 10^3$/mcL ($4.5–11.0 \times 10^9$/L)	
Red blood cell count	$4.0–5.5 \times 10^6$/mcL ($4.0–5.5 \times 10^{12}$/L)	
Hemoglobin	12.0–17.4 g/dL (120–174 g/L)	
Hematocrit	36–52% (0.36–0.52 L/L)	
Mean cell volume	80–100 fL	
Mean cell hemoglobin	28–34 pg	
Mean cell hemoglobin concentration	32–36 g/dL	
Red cell distribution width	11.5–14.5%	
Reticulocyte count		
Relative (%)	0.5–2.0%	
Absolute ($\times 10^9$/L)	$25–75 \times 10^9$/L	
Platelet count	$150–400 \times 10^9$/L	
Mean platelet volume	6.8–10.2 fL	
Automated WBC Differential	Relative (%)	Absolute ($\times 10^9$/L)
Neutrophils	40–80	1.8–7.0
Lymphocytes	25–35	1.0–4.8
Monocytes	2–10	0.1–0.8
Eosinophils	0–5	0–0.4
Basophils	0–1	0–0.2

[a] Male and female reference intervals are combined. For age- and sex-specific reference intervals, see the front cover of this textbook. Additional parameters are dependent upon instrumentation (Chapter 39). Data are shown as conventional units (SI units).

Leukocyte Count, Erythrocyte Count, Hematocrit, and Hemoglobin

The leukocyte count (WBC count), erythrocyte count (RBC count), hematocrit (Hct), and hemoglobin (Hb) are determined using automated instrumentation (Table 10-2). The WBC and RBC counts are reported as the number of cells per liter. The WBCs are reported as billions of cells per liter ($\times 10^9$/L), and the RBCs are reported as trillions of cells per liter ($\times 10^{12}$/L). The **hematocrit** measures the volume that the RBCs occupy within whole blood and is reported as a percentage (%) or as the volume of RBCs in liters divided by the volume of whole blood in liters [L/L] (Chapter 37). In automated analyzers, the hematocrit is usually calculated from the measured MCV and RBC count using the following formula (Chapter 39):

$$\text{Hct (L/L)} = \frac{\text{MCV (fL)} \times \text{RBC count } (\times 10^{12}\text{/L})}{1000}$$

The hemoglobin is measured spectrophotometrically after it has been released from lysed erythrocytes. It is reported in grams per deciliter or grams per liter.

The laboratory professional interprets the accuracy of the RBC count, hematocrit, and hemoglobin values using a quick mathematical check called the **rule of three**. Simply, the RBC count $\times$ 3 = hemoglobin $\times$ 3 = hematocrit (%). If the calculated values do not agree within $\pm$3% of the measured values, a measurement error or instrument malfunction could have occurred, or the patient could have a pathology that requires investigation. Potential underlying problems and interfering substances associated with such a mismatch are described in Chapter 43, Table 43-8 and Table 43-9.

A diurnal variation in blood cell concentration occurs in which the value for the WBC count is lowest in the morning and highest in the afternoon, whereas the RBC count, Hct, and Hb are just the opposite: higher values are observed in the morning.[4]

☑ CHECKPOINT 10-2

The results on a blood specimen drawn from a patient in the doctor's office and transported to the hospital laboratory were: Hb 15 g/dL, Hct 35%, and RBC 2.8 $\times 10^{12}$/L. Should these results be reported? What should be the next step?

Erythrocyte Indices

The erythrocyte indices help classify the erythrocytes by their size and hemoglobin content (Chapter 37). Hemoglobin, hematocrit, and erythrocyte count values are used to calculate the three indices: **mean cell volume (MCV)**, **mean cell hemoglobin (MCH)**, and **mean cell hemoglobin concentration (MCHC)** (Table 10-2). When calculating the indices, it is important to note that the conversion factors used in the formulas vary depending on the use of conventional units or Systeme International Units (SI) for hemoglobin and hematocrit. Table 37-9 provides both conversion factors (Chapter 37). These indices suggest how the RBCs will appear microscopically and provide significant diagnostic information (most commonly for the diagnosis of anemias). Laboratory professionals correlate the indices with the Hct, Hb, and RBC count to ensure that technical problems are identified when they occur (see Chapter 43, Tables 43-8 and 43-9 for factors that falsely increase or decrease the RBC indices).

Mean Cell Volume

The MCV denotes the average volume of individual erythrocytes and is expressed in femtoliters (fL, 10^{-15} L). It is measured by automated instrumentation (Chapter 39) and can be calculated from the Hct and RBC count.

$$\text{MCV (fL)} = \frac{\text{Hct (L/L)} \times 1000}{\text{RBC count } (\times 10^{12}\text{/L})}$$

★ **TABLE 10-3** Classification of Erythrocytes Based on MCV

Terminology	Description
Normocytic	80.0–100.0 fL
Microcytic	Red cells with a reduced volume (<80 fL)
Macrocytic	Red cells with an increased volume (>100 fL)
Anisocytosis	Increased variation in the range of red cell sizes

The MCV is used to classify cells as normocytic, microcytic, or macrocytic (Table 10-3 ★) and usually correlates with the appearance of cells on stained blood smears (i.e., cells with an increased MCV appear larger [macrocytic], and cells with a decreased MCV appear smaller [microcytic]). However, it must be remembered that the MCV is a measurement of volume, whereas estimation of the size of flattened cells on a blood smear is a measurement of cell diameter. Cell diameter and cell volume are not the same.

EXAMPLE

A patient has an Hct of 0.45 L/L and an RBC count of 5.0×10^{12}/L.

$$90.0 \text{ fL} = \frac{0.45 \times 1000}{5}$$

The value, 90.0 fL, indicates that the cell has a volume that falls within the reference interval (80–100 fL) and is therefore classified as **normocytic**.

Spherocytes (discussed later in this chapter and in Chapters 17 and 19) usually have a normal or only slightly decreased volume (MCV), but on a stained smear, they cannot flatten as much as normal erythrocytes because of a decreased surface area and increased rigidity. Spherocytes, therefore, often appear to have a smaller diameter than normal cells. On the other hand, codocytes (discussed later in this chapter and in Chapter 11) can appear larger due to an increased diameter, but the MCV is often normal. Generally, abnormalities in the MCV are clues to disease processes of the hematopoietic system.

Mean Cell Hemoglobin

The MCH is a measurement of the average weight (in picograms, 10^{-12} g) of hemoglobin in individual erythrocytes. The MCH is calculated from the hemoglobin and erythrocyte count.

$$\text{MCH (pg)} = \frac{\text{Hb (g/dL)} \times 10}{\text{RBC } (\times 10^{12}\text{/L})}$$

EXAMPLE

A patient has an Hb concentration of 15 g/dL and an RBC count of 5.0×10^{12}/L.

$$30 \text{ pg} = \frac{15 \times 10}{5}$$

The value, 30.0 pg, indicates that the RBCs contain an average weight of hemoglobin that is within the reference interval (28.0–34.0 pg).

The MCH does not take into account the size of a cell; it should not be interpreted without taking into consideration the MCV because the MCH varies in a direct linear relationship with the MCV. Cells with less volume typically contain less hemoglobin while cells with larger volume typically contain more hemoglobin.

Mean Cell Hemoglobin Concentration

The MCHC is the ratio of hemoglobin mass to volume in which it is contained (i.e., average concentration of hemoglobin in a deciliter of erythrocytes, expressed in g/dL). The MCHC is calculated from the Hb and Hct.

$$\text{MCHC (g/dL)} = \frac{\text{Hb (g/dL)}}{\text{Hct (L/L)}}$$

EXAMPLE

A patient has an Hb concentration of 15 g/dL and an Hct of 0.45 L/L.

$$33.3 \text{ g/dL} = \frac{15}{0.45}$$

The value, 33.3 g/dL, reveals that the cells contain a normal concentration of hemoglobin (32.0–36.0 g/dL) and are therefore **normochromic**.

The MCHC indicates the concentration of hemoglobin in the general cell population and is described by the suffix *-chromia*, meaning color (Table 10-4 ★). Cells can be classified morphologically as hypochromic if the area of central pallor is >1/3 of the cell size. The term *hyperchromic* should be used sparingly (if ever). The only erythrocyte that is hyperchromic with an MCHC of >36.0 g/dL is the spherocyte. Spherocytes have a decreased surface-to-volume ratio due to a loss of membrane but have not lost an appreciable amount of their hemoglobin. In certain conditions, the indices MCV, MCH, and MCHC can be falsely elevated. This is discussed in Chapter 43.

Red Cell Distribution Width

Because the MCV represents an average of erythrocyte volume, it is less reliable in describing the erythrocyte population when considerable variation in erythrocyte volume/size (anisocytosis) occurs. The **red cell distribution width (RDW)** (Table 10-2) is the coefficient of variation of the MCV and may be referred to as the RDW-coefficient of variation (RDW-CV). The formula for the RDW-CV, a calculated index from hematology instruments to help identify anisocytosis, follows:

$$\text{RDW-CV} = \frac{1 \text{ standard deviation (SD) of MCV}}{\text{MCV}} \times 100$$

★ **TABLE 10-4** Classification of Erythrocytes Based on MCHC

Normochromic	32.0–36.0 g/dL
Hypochromic	<32.0 g/dL
Hyperchromic	>36.0 g/dL

Abnormal increased RDW values (>14.5%) indicate an increase in the heterogeneity of erythrocyte size. No known abnormalities are represented by a decreased RDW.

Caution must be used in interpreting the RDW-CV because it reflects the ratio of the standard deviation of cell volume and the MCV. An increased standard deviation (heterogeneous cell population) with a high MCV can give a normal RDW-CV. Conversely, a normal standard deviation (homogenous cell population) with a low MCV can give an increased RDW-CV. Examination of the erythrocyte histogram and stained blood smear gives clues as to the accuracy of the RDW-CV in these cases. When the standard deviation is increased, indicating a true variability in cell size, the base of the erythrocyte histogram is broader than usual. Because of this interpretation issue, automated instruments often report the RDW-CV and RDW-standard deviation (RDW-SD). The RDW-SD is directly measured and not affected by the MCV (detailed information in Chapter 39).

CASE STUDY *(continued from page 155)*

1. Calculate the Hct, MCH, and MCHC from the initial results.
2. Evaluate the calculated Hct, MCH, and MCHC as compared with the reference intervals for a 48-year-old female.

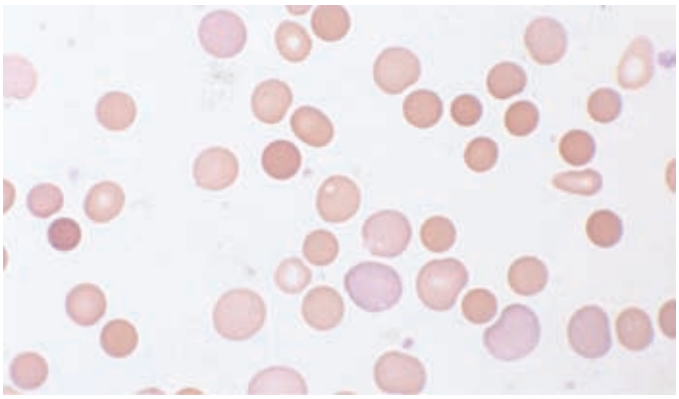
☑ CHECKPOINT 10-3

A patient has an MCV of 130 fL and an RDW of 14.5. Review of the blood smear reveals anisocytosis. Explain the discrepancy between the blood smear finding and RDW. Why is examination of an erythrocyte histogram/cytogram helpful in this case?

Reticulocyte Count

Immature, anuclear erythrocytes containing organelles and residual ribosomes for hemoglobin synthesis are known as *reticulocytes* (Chapter 5), which usually spend 2–3 days in the bone marrow and an additional day in the peripheral blood before their RNA is degraded and they become mature erythrocytes. The peripheral blood reticulocyte count indicates the degree of effective bone marrow activity and is one of the most useful and cost-effective laboratory tests in monitoring response to therapy and pathophysiology of anemia (Chapters 11 and 37). Reticulocytes can sometimes be identified as *polychromatophilic* erythrocytes (erythrocytes with a bluish tinge) on Romanowsky-stained smears. The polychromatophilia is due to the presence of basophilic RNA within ribosomes mixed with acidophilic hemoglobin (Figure 10-1 ■).

A supravital stain such as new methylene blue or brilliant cresyl blue must be used to definitively identify the presence of reticulocytes (Figure 10-2 ■). Although automated methods for reticulocyte enumeration are available on some hematology instruments, many laboratories use a manual method (Chapter 37). Test results are expressed as a percentage of reticulocytes in relation to the total RBC count (relative count) or as the absolute number (see the following section).

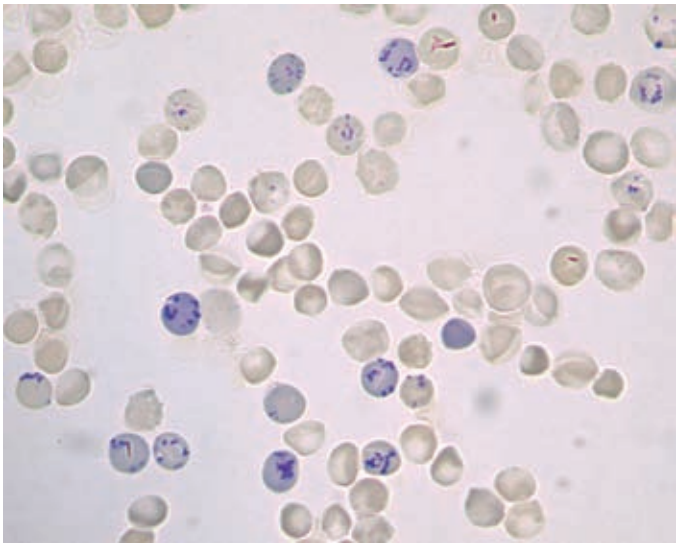


■ **FIGURE 10-1** The large erythrocytes with a bluish tinge are polychromatophilic erythrocytes, which are larger than the more mature erythrocytes. Note also the spherocytes (peripheral blood; Wright-Giemsa stain; 1000× magnification).

In the automated method, >30,000 RBCs are assessed, so the method is more precise than the manual method (which assesses only 1000 RBCs) and is more accurate when the reticulocyte count is very low.

Absolute Reticulocyte Count

The absolute reticulocyte count is a more informative index of erythropoietic activity than the relative reticulocyte count (Table 10-2). When reported as a percentage, the reticulocyte count does not indicate the relationship between the peripheral blood erythrocyte mass and the number of reticulocytes being produced. The reticulocyte count reported as a percentage can appear increased because of either an increase in the number of reticulocytes in the circulation *or* a decrease in the number of total RBCs. Therefore, it is recommended that in addition to the percentage of reticulocytes, laboratory professionals report the absolute reticulocyte count to provide a more useful estimate of reticulocyte production. Automated analyzers can provide



■ **FIGURE 10-2** The erythrocytes with the particulate inclusions are reticulocytes. The inclusions represent reticulum that stains with the supravital stain brilliant cresyl blue (peripheral blood; 1000× magnification).

the absolute count and can be calculated when using manual methods for reticulocytes:

$$\text{Absolute reticulocyte } (\times 10^9/\text{L}) = \text{RBC count } (\times 10^{12}/\text{L}) \times \text{Reticulocyte count (\%)}$$

EXAMPLE

A patient has an RBC count of 3.5×10^{12}/L and a 10% reticulocyte count.

$$350 \times 10^9/\text{L} = 3.5 \times 10$$

The value 350×10^9/L represents an increase in reticulocyte production since the mean normal value is 90×10^9/L.

Platelet Count and Mean Platelet Volume

Automated hematology instruments generate the platelet count, which is reported as billions of platelets per liter (number of platelets $\times 10^9$/L). The mean platelet volume (MPV) is similar to the MCV for erythrocytes because it represents the average volume of individual platelets. The laboratory professional utilizes both the platelet count and the MPV to assess thrombopoiesis and pathologic conditions related to platelets (Chapters 9 and 33). A decreased platelet count generally represents decreased thrombopoiesis, increased platelet destruction, or consumption. Reactive or malignant conditions can cause an increase in the platelet count.

WBC Differential

The WBC differential is an analysis and enumeration of the various subtypes of WBCs (Chapter 7). An altered concentration of one specific type of leukocyte most commonly causes an increase or decrease in the total WBC count. For this reason, an abnormal total WBC count should be followed by a WBC differential, also known as a *diff*. The WBC differential (Table 10-2) can be performed by automated instruments or manually. To perform a manual WBC differential, a blood smear stained with a Romanowsky-type stain (usually Wright's stain) is viewed microscopically. A total of 100 leukocytes are viewed and each leukocyte subtype is classified. The differential results are reported as the percentage of each cell type counted. To accurately interpret whether an increase or decrease in cell types exists, the absolute concentration of each cell type is calculated using the results of the WBC count and the differential (see Chapter 7 for the calculation for absolute WBC values). A detailed discussion of the automated WBC differential is found in Chapter 39.

☑ CHECKPOINT 10-4

A CBC was performed on a blood specimen from a 15-year-old female. The results were: Hb 13 g/dL, Hct 0.40 L/L, RBC 4.5×10^{12}/L, WBC 15×10^9/L. The differential revealed 70% segmented neutrophils, 25% lymphocytes, 4% monocytes, 1% eosinophils. Calculate the indices and absolute WBC differential counts. Are any of these parameters outside the reference intervals for this patient? If so, which ones?

The Peripheral Blood Smear

Each testing location and institution sets the parameters that trigger the necessity of a manual morphologic examination of the peripheral smear, but generally a peripheral blood smear is prepared for microscopic examination when CBC values obtained from an automated instrument differ from what is considered normal (Table 10-2). Instructions on how to prepare and stain a peripheral blood smear are discussed in Chapter 37. A laboratory professional reviews the blood smear for overall quality, the morphology of white blood cells, red blood cells, and platelets, and performs a WBC differential. The peripheral smear is correlated with the parameters reported by the instrument as the culminating interpretation of the CBC.

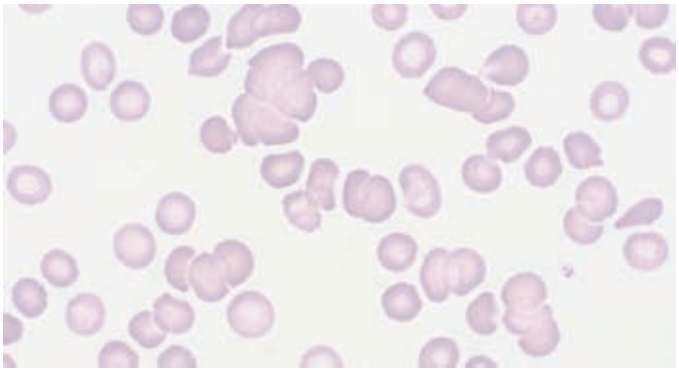
Briefly, a "wedge smear" is made by placing a small drop of blood at one end of a microscope slide and spreading that drop to create a thin smear or "film" of blood. After the blood has dried, the slide is stained using a Romanowsky-type stain (Chapter 37). A well-made and properly stained blood smear is required for accurate interpretation of the CBC. The slide is examined macroscopically and microscopically to ensure that the blood was spread and stained properly. The optimal blood smear is pinkish purple in color and transitions to a feathered edge (summarized in Tables 37-2 and 37-4). Information such as RBC agglutination (appears as a grainy blood smear), lipidemia (represented as holes within the smear), and multiple myeloma (bluish-colored smear) can be suspected during this important macroscopic evaluation of the blood smear and should be noted by the laboratory professional before moving on to the microscopic evaluation. This macroscopic assessment determines whether the blood smear is acceptable for microscopic analysis (described in the next section).

Low-Power Magnification

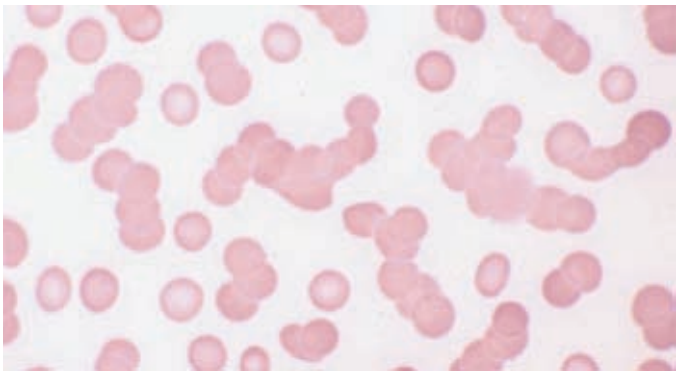
The laboratory professional first assesses the general appearance and distribution of WBCs, RBCs, and platelets using low power magnification 10× objective (100× magnification; Table 10-5 ★).

★ **TABLE 10-5** Summary of the Microscopic Peripheral Blood Smear Examination

	10× Objective (100× magnification)	40× Objective (400× magnification)	100× Objective (1000× magnification)
WBCs	Scan for abnormal or large cells Smudge cells	Perform WBC estimate	Evaluate leukocyte morphology Perform 100-cell differential
RBCs	Scan for rouleaux and agglutination	Determine the critical area	Evaluate erythrocyte morphology: Assess size, shape, color, presence of inclusions
Platelets	Scan for clumps and satellitism		Perform platelet estimate Evaluate platelet morphology: Assess size and granularity



■ **FIGURE 10-3** This blood smear is from a patient with cold agglutinin disease. Notice the clumping of the erythrocytes (peripheral blood; Wright-Giemsa stain; 1000× magnification).



■ **FIGURE 10-4** The erythrocytes are stacked on top of one another like a stack of coins (rouleaux). This blood smear is from a patient with multiple myeloma, a malignant plasma cell disorder. The cells stack because of the large amount of protein in the plasma (peripheral blood; Wright-Giemsa stain; 1000× magnification).

The properties of a well-made smear are described in Table 37-4. A high concentration of WBCs at the furthest edge of the smear (the feathered edge) indicates poor cell distribution and is sufficient evidence that a new smear should be made. In addition, very large or abnormal WBCs are often pushed to the outer edges of the smear. Cells that have ruptured are called *smudge cells*. These are often B lymphocytes and their presence is characteristic of pathological conditions such as chronic lymphocytic leukemia. The laboratory professional also performs an estimate of the number of WBCs (i.e., WBC estimate) under low power (either 100× or 400× magnification) and correlates the WBC estimate to the WBC count (Chapter 37, Figure 37-6).

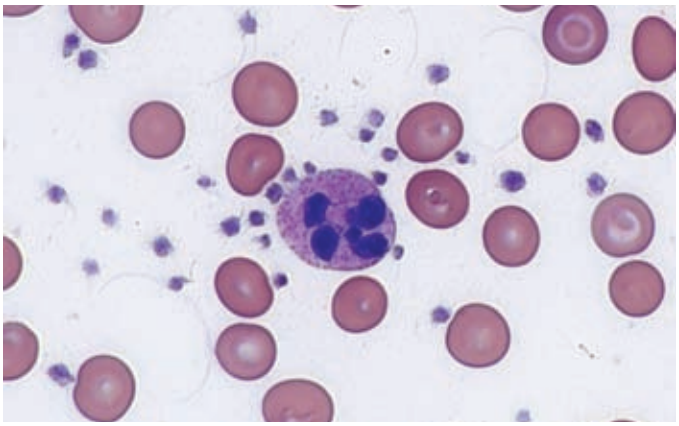
On a well-made blood smear, the erythrocytes are evenly distributed and well separated on the feathered edge of the smear. Stacking or aggregating of cells is associated with certain pathologic states (Chapter 11 and Table 10-6 ★). In the presence of IgM antibodies (cold agglutinins) directed against erythrocyte antigens, erythrocytes can agglutinate forming irregular clusters of varying sizes (Figure 10-3 ■). This *agglutination* forms irregular, grapelike clusters that are readily differentiated from rouleaux. On automated hematology analyzers, a CBC with an elevated MCV and low RBC count but a normal hemoglobin suggests the presence of cold-reacting erythrocyte agglutinins. In addition, the calculated hematocrit will be falsely decreased and the MCH and MCHC will be falsely increased. The effect of cold agglutinins is overcome by keeping the blood at 37 °C. When performing blood counts, the diluting fluid also must be kept at 37 °C.

Rouleaux is an alignment of erythrocytes one on top of another resembling a stack of coins (Figure 10-4 ■). This phenomenon occurs normally when blood is collected and allowed to stand in tubes. It can also be seen in the thick portion of blood smears. In certain pathologic states that are accompanied by an increase in fibrinogen or globulins, rouleaux becomes marked and is readily seen in the feathered edge of blood smears. When the erythrocyte assumes abnormal shapes, such as sickled forms, rouleaux formation is inhibited. Rouleaux is also inhibited when erythrocytes are suspended in saline. The presence of rouleaux or agglutination are possible indications that a new smear should be made.

The platelets must also be evaluated using low power magnification. The manner in which the platelets have spread on the slide is checked because platelet clumps[5] can be pushed to the outer edge of the smear, and in some cases, platelets can adhere to neutrophils (a phenomenon called *satellitism*, Figure 10-5 ■).[6] This can result in falsely decreased estimation of the platelet count. Platelet clumps and

★ **TABLE 10-6** Abnormalities in Erythrocyte Arrangement

Terminology	Description	Associated Physiologic State
Agglutination	Irregular clumps of red blood cells	Due to antigen–antibody interaction
Rouleaux	Red blood cells arranged in rolls or stacks	Usually associated with abnormal or increased plasma proteins (red blood cells can be dispersed by mixing cells with saline)



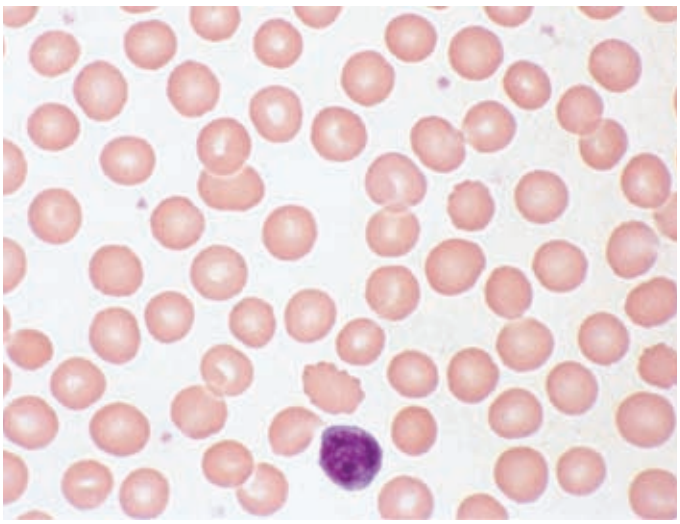
■ **FIGURE 10-5** Platelet satellitism. The neutrophil in the center is surrounded by platelets (peripheral blood; Wright-Giemsa stain; 1000× magnification).

satellitism can be eliminated using sodium citrate as an anticoagulant (Chapter 37, Table 37-6). Finally, fibrin strands can be observed in this scan of the blood smear, indicating that the blood sample was coagulated (likely due to improper mixing following the venipuncture). In either case, a new sample should be obtained from the patient and a new smear should be made. Other common sources of error that warrant morphologic examination of a peripheral blood smear can be found in Chapters 37 and 43.

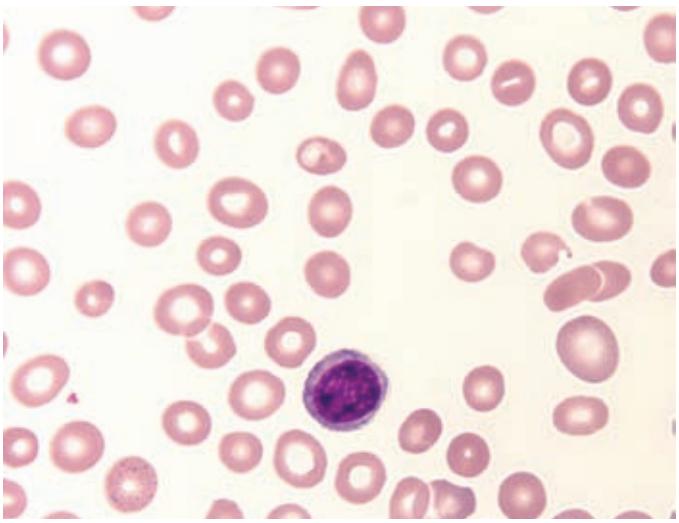
The final task at low power magnification is to determine the **critical area** of the smear that will be used to perform the morphologic examination of cells. This critical area is usually identified using the 40× objective (400× magnification) and is characterized by the proximity of RBCs to each other (the area of the smear in which very few RBCs overlap or touch and are generally distributed in a uniform manner). At high power magnification, the critical area is used to evaluate RBC morphology and perform the WBC differential and platelet estimate (Figure 10-6a ■).

High-Power Magnification

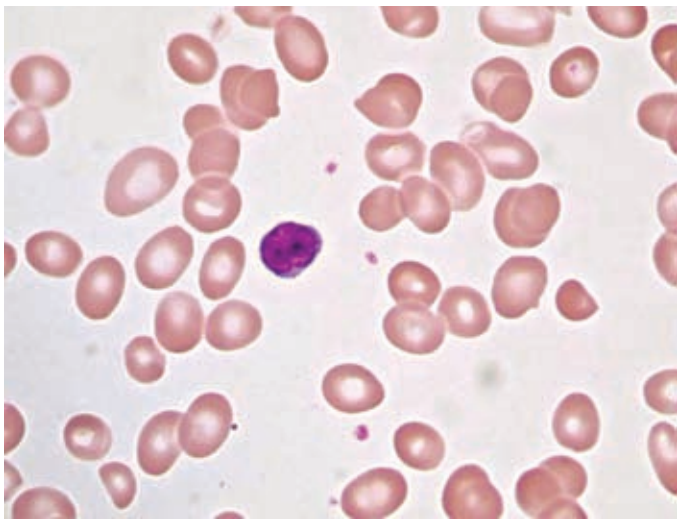
Following the quick, yet important, scan of the blood smear on low magnification, the laboratory professional evaluates the smear on high power, often at 1000× magnification. Ultimately, interpretation of the microscopic findings in the peripheral blood smear and correlation, or lack thereof, with the automated instrument report is made for all parameters of the CBC. A WBC differential is performed in which 100 cells are observed and classified to determine the relative number of leukocytes as a percentage (Chapter 7) and to identify the presence of morphologic abnormalities (Table 10-7 ★, Chapters 21



a



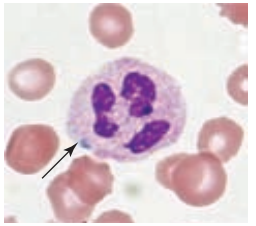
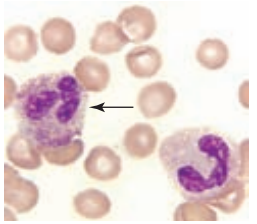
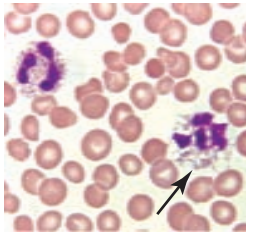
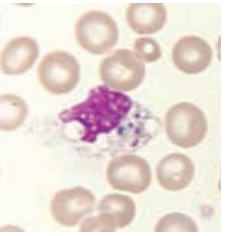
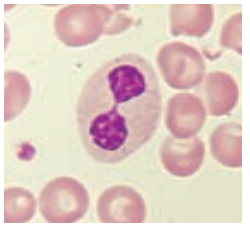
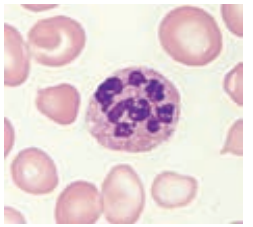
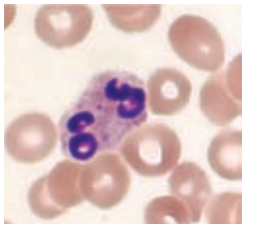
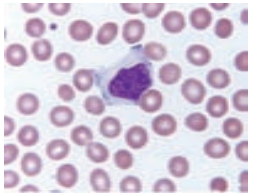
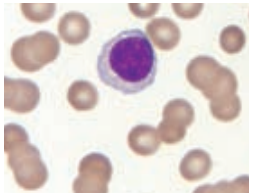
b



c

■ **FIGURE 10-6** **(a)** Normocytic, normochromic erythrocytes. Compare the size of the cells to the nucleus of the lymphocytes. **(b)** The erythrocytes are microcytic (much smaller than the lymphocyte nucleus). **(c)** The erythrocytes are macrocytic (much larger than the lymphocyte nucleus) (peripheral blood; Wright-Giemsa stain; 1000× magnification).

★ TABLE 10-7 Morphologic Abnormalities of WBCs (Nonmalignant)

Neutrophil cytoplasmic morphology	Dohle bodies	Toxic granulation	Vacuoles	Intracellular organisms
Associated Disease State	May Hegglin anomaly, infection	Infection	Infection	Infection
Image				
Neutrophil nuclear morphology	Bilobed or round nuclei	Nuclear hypersegmentation	Pyknotic nuclei	
Associated Disease State	Pelger-Huet anomaly	Megaloblastic anemia	Necrobiotic (dying) neutrophil	
Image				
Lymphocyte morphology	Reactive cells	Plasmacytoid		
Associated Disease State	Viral infection	Infection		
Image				

[a]See Chapters 21 and 22.

and 22). A platelet estimate is performed (Chapter 37, Figure 37-7) and compared with the instrument-generated platelet count; the morphology of the platelets is noted. Finally, the RBC morphology is assessed for size, shape, color, and inclusions using either the 40× or 50× objective and compared with the instrument report for the RBC indices. To evaluate abnormalities including inclusions, the laboratory professional should review the slide with the 100× objective (1000× magnification).

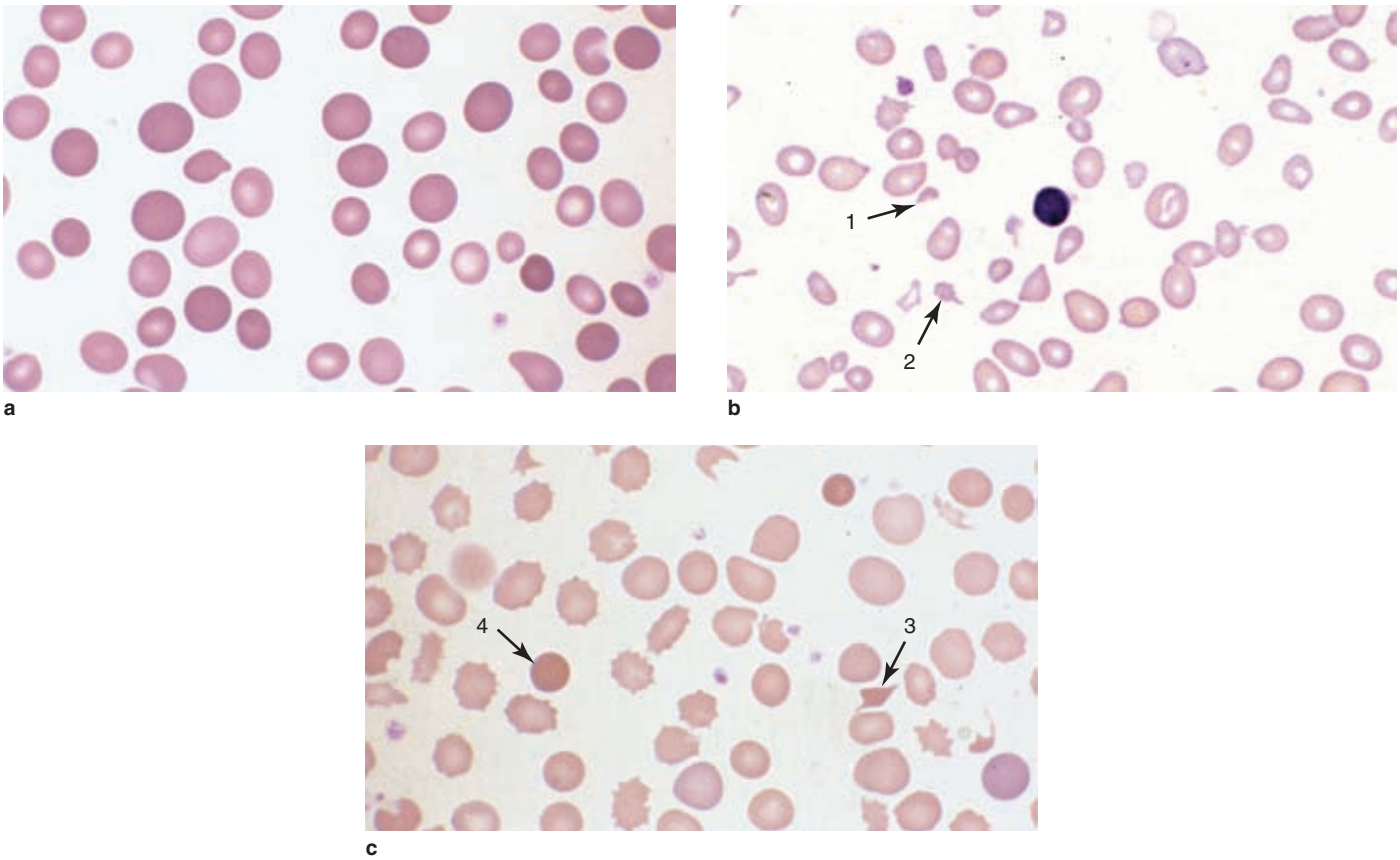
Erythrocyte Morphology

The erythrocyte is sometimes called a *discocyte* because of its biconcave shape. On a Romanowsky-stained blood smear, the erythrocyte appears as a disc with a central area of pallor surrounded by a rim of pink-staining hemoglobin (the center stains lighter in color compared with the rim). The area of pallor is caused by the closeness occurring between the two concave portions of the membrane when the cell becomes flattened on a glass slide. Normally the area of pallor occupies about one-third the diameter of the cell.

Anisocytosis denotes a nonspecific variation in the size of the cells. Some variation in size is normal because of the variation in age of the erythrocytes with younger cells being larger and older cells smaller (Figures 10-7a and b ■). **Poikilocytosis** is the general term used to describe a nonspecific variation in the shape of erythrocytes (Figures 10-7b and 10-7c). It is important to note that some abnormal morphology can be artifactual because of poorly made or improperly stained smears.

Anisocytosis

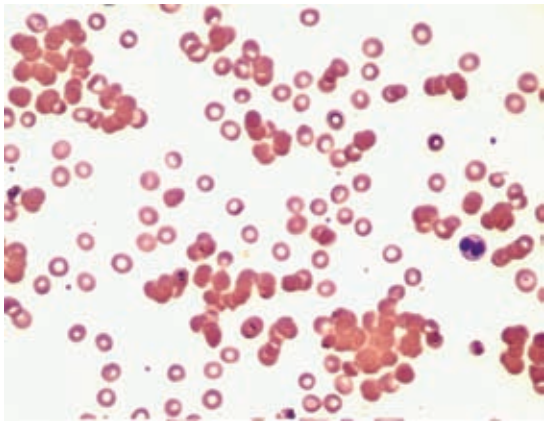
Anisocytosis can be detected by examining the blood smear and/or by reviewing the MCV and RDW (discussed earlier in this chapter). Normal erythrocytes have a diameter of about 7–8 mcM (μm) and an MCV of 80–100 fL. If the majority of cells are larger than normal, they are macrocytic; if smaller than normal, they are microcytic (Table 10-3). If there is a significant variation in size with microcytic, normocytic, and macrocytic cells present, the MCV can fall within the reference interval because it is an average of cell volume. In this case, the RDW is helpful. An RDW of >14.5% suggests that the erythrocytes are heterogeneous in size, which makes the MCV less reliable. Microscopic examination of the cells is especially helpful when the RDW is elevated. To evaluate erythrocyte size microscopically, the



■ **FIGURE 10-7** **(a)** Erythrocytes with anisocytosis. **(b)** Erythrocytes with marked anisocytosis and poikilocytosis. Arrows point to a schistocyte (1) and an acanthocyte (2). **(c)** Poikilocytosis with acanthocytes, helmet cell, elliptocytes, echinocytes, schistocytes (3), and spherocytes (4). There is also anisocytosis with microcytes and macrocytes. At least two of the macrocytes are polychromatophilic (peripheral blood; Wright-Giemsa stain; 1000× magnification).

CASE STUDY *(continued from page 159)*

Because of the abnormal values found in the CBC, the laboratory professional made a blood smear and performed a WBC differential and complete evaluation of the RBCs and platelets. The primary abnormal finding is seen in the following smear image.



3. What should the laboratory professional report about the RBCs in the critical area of this smear?
4. Which results of the CBC might be affected by the findings on the smear?
5. Explain why the abnormal values in the CBC occurred in this case.
6. Predict the RDW value for the RBCs from this patient as increased or normal.

cells are compared with the nucleus of a normal small lymphocyte. Normocytic erythrocytes are about the same size as the lymphocyte nucleus (Figure 10-6a). Figures 10-7a and b show erythrocytes with a marked degree of anisocytosis.

Microcytes **Microcytes** are erythrocytes with a diameter of <7.0 mcM and are present when the MCV is <80 fL (Figure 10-6b). The cell is usually hypochromic but can be normochromic. Microcytes in the shape of spheres (microspherocytes) can appear hyperchromic.

Macrocytes **Macrocytes** are larger than normal erythrocytes with a diameter >8.0 mcM and are present when the MCV is >100 fL (Figure 10-6c). The cell usually contains an adequate amount of hemoglobin resulting in a normal MCHC and normal to increased MCH. Young erythrocytes are normally larger than mature erythrocytes, but within a day of entering the blood stream, the spleen grooms them to a normal size. When the reticulocyte count is increased, the MCV can be increased.

☑ CHECKPOINT 10-5

Results from a CBC include MCV 63 fL, MCH 16.7 pg, and MCHC 26.5 g/dL. Describe these cells.

Poikilocytosis

Most laboratories report only significant poikilocytosis. The stained smear should be reviewed while keeping in mind the overall context of the laboratory results and the significance of the reported findings. To determine the significance of and to decide whether to report poikilocytes, the following should be considered:

1. Will it assist in differential diagnosis of the disease (likely anemia)?
2. Will it make a difference in the management of the patient?
3. Is the dominant poikilocyte significant in this setting?
4. Do the specific constellation of findings indicate a particular pathologic state?

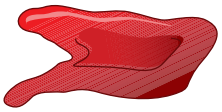
Figure 10-6a illustrates normal erythrocytes, and Figure 10-7b and c illustrate poikilocytosis. Following is a description of specific types of poikilocytes (Table 10-8 ★).

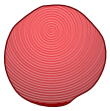
Acanthocytes **Acanthocytes**, also called *spur cells*, are small spherical cells with irregular thornlike projections (Figure 10-7b, Table 10-8). Often the projections have small bulblike tips. Acanthocytes do not have a central area of pallor. These cells have membranes with free cholesterol accumulating preferentially in the outer bilayer of the membrane leading to decreased fluidity. Remodeling by the spleen results in spheroidal cells with irregular surface projections. These cells are readily trapped in the spleen.

Codocytes **Codocytes**, also called **target cells**, are thin, bell-shaped cells with an increased surface-to-volume ratio (Table 10-8, Figure 10-8 ■). On stained blood smears, the cells have the appearance of a target with a bull's-eye in the center. An achromic zone and a thin outer ring of pink-staining hemoglobin surround the bull's eye. The typical appearance of these cells is discernible in the area of the slide only where the cells are well separated but not in the extreme outer-feathered edge where all cells are flattened. Target cells can appear as artifacts when smears are made in a high-humidity environment or when a wet smear is blown dry rather than fan dried. Target cells have an increased surface-to-volume ratio of the cell.

Dacryocytes **Dacryocytes**, also called **teardrops**, are erythrocytes that are elongated at one end to form a teardrop or pear-shaped cell (Table 10-8 and Figure 10-9 ■). The teardrop morphology can form after erythrocytes containing cellular inclusions have traversed the

★ TABLE 10-8 Erythrocyte Morphologies

Terminology	Synonyms	Description	Associated Disease States	Drawing of Morphology
Poikilocytosis	—	Increased variation in the shape of red cells	See disease states associated with specific poikilocytes on this table	
Acanthocyte (spike)	Spur cell	Red cells with spicules of varying length irregularly distributed over the surface; no area of pallor	Abetalipoproteinemia; alcoholic liver disease; disorders of lipid metabolism; post splenectomy; fat malabsorption; retinitis pigmentosa, Chapters 15, 17	
Codocyte (bell)	Target cell	Thin, bell-shaped, with increased surface-to-volume ratio; on stained blood smears, appears as a target with a central bull's-eye, surrounded by achromic zone and outer ring of hemoglobin	Hemoglobinopathies; thalassemias; obstructive liver disease; iron deficiency anemia; splenectomy; renal disease; LCAT deficiency, Chapters 12, 14, 15	
Dacryocyte (tear)	Teardrop	Round cell with a single elongated or pointed extremity; may be microcytic and/or hypochromic	Myelophthisic anemias; primary myelofibrosis (PMF); thalassemias, Chapter 14	
Drepanocyte (sickle)	Sickle cell	Contain polymerized hemoglobin showing various shapes: sickle, crescent, or boat shaped	Sickle cell disorders, Chapter 13	
Echinocyte (sea urchin)	Burr cell; crenated cell	Spiculated red cells with short equally spaced projections over the entire surface	Liver disease; uremia; pyruvate kinase deficiency; peptic ulcers; cancer of stomach; heparin therapy, Chapters 15, 18	
Elliptocyte (oval)	Ovalocyte; pencil cell; cigar cell	Oval to elongated ellipsoid cell with central area of pallor and hemoglobin at both ends	Hereditary elliptocytosis; iron deficiency anemia; thalassemia; anemia associated with leukemia, Chapters 12, 14, 17	
Keratocyte (horn)	Helmet cell; horn-shaped cell	Red cells with one or several notches with projections that look like horns on either end	Microangiopathic hemolytic anemias; heart-valve hemolysis; Heinz-body hemolytic anemia; glomerulonephritis; cavernous hemangiomas, Chapters 13, 20;	
Knizocyte	—	RBC with more than two concavities; on stained blood smears has a dark band of hemoglobin across the center with a pale area on either side	Conditions in which spherocytes are found, Chapter 17	

Terminology	Synonyms	Description	Associated Disease States	Drawing of Morphology
Leptocyte (thin)	Thin cell	Thin, flat cell with hemoglobin at periphery; usually cup-shaped, MCV is decreased but cell diameter is normal	Thalassemia; iron deficiency anemia; hemoglobinopathies; liver disease, Chapters 13, 14,15, 17	
Schistocyte (cut)	Schizocyte; fragmented cell	Fragments of red cells; variety of shapes including triangles, commas; microcytic	Microangiopathic hemolytic anemias; heart-valve hemolysis; disseminated intravascular coagulation; severe burns; uremia, Chapter 20	
Spherocyte	—	Spherocytic red cells with dense hemoglobin content (hyperchromatic); lack an area of central pallor	Hereditary spherocytosis; immune hemolytic anemias; severe burns; transfusion with ABO incompatibility; Heinz-body hemolytic anemias, Chapters 13, 17, 19, 20	
Stomatocyte (mouth)	Mouth cell; cup form; mushroom cap	Uniconcave red cells with the shape of a very thick cup; on stained blood smears cells have an oval or slitlike area of central pallor	Hereditary stomatocytosis; spherocytosis; alcoholic cirrhosis; anemia associated with Rh null disease; lead intoxication; neoplasms, Chapters 17, 20	

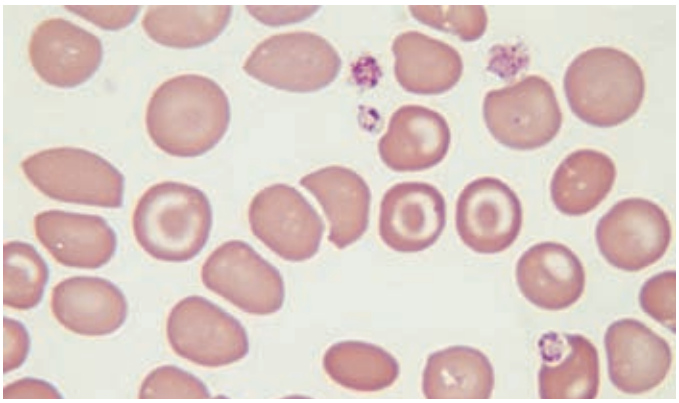


FIGURE 10-8 Codocytes, also called target cells (peripheral blood; Wright-Giemsa stain; 1000× magnification).

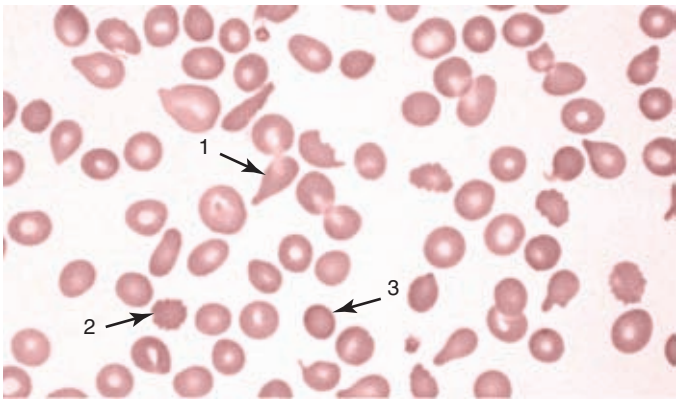
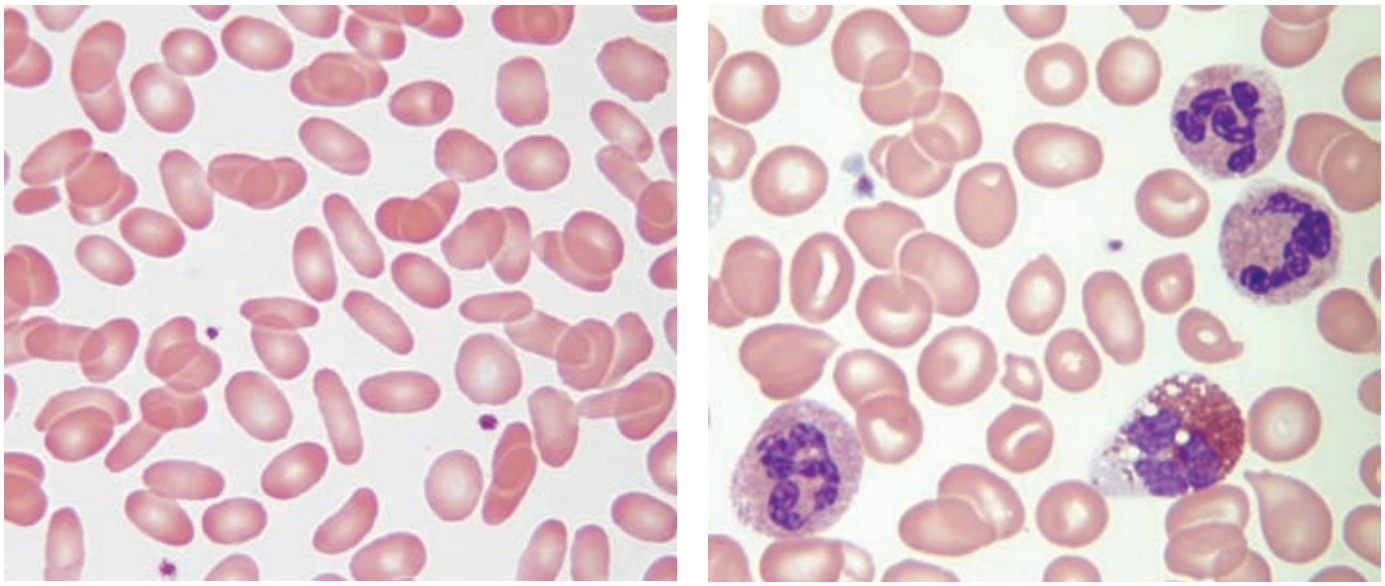


FIGURE 10-9 Note the presence of dacryocytes (teardrops) (1). Note also the echinocytes (2), acanthocytes, and spherocytes (3) (peripheral blood; Wright-Giemsa stain; 1000× magnification).

spleen. Erythrocytes with inclusions are more rigid in the area of the inclusion, and this portion of the cell has more difficulty passing through the splenic filter than the rest of the cell. As splenic macrophages attempt to remove this rigid inclusion, the cell is stretched into an abnormal shape. The teardrop cannot return to its original shape because the cell either has been stretched beyond the limits of deformability of the membrane or has remained in the abnormal shape for too long.

Sickle Cells **Sickle cells**, also called **drepanocytes**, are elongated, crescent-shaped erythrocytes with pointed ends (Table 10-8, Chapter 13). Some forms have more rounded ends with a flat rather



a b

■ **FIGURE 10-10** Note the morphologic differences between **(a)** elliptocytes and **(b)** ovalocytes (peripheral blood; Wright-Giemsa stain; 1000× magnification).

than concave side. These modified forms of sickle shape can be capable of reversing to the normal discocyte. Sickle cell formation can be observed in stained blood smears from patients with sickle cell anemia. The hemoglobin within the cell is abnormal and polymerizes into rods at decreased oxygen tension or decreased pH. The cell first transforms into a holly leaf shape and as the hemoglobin polymerization continues, it transforms into a sickle-shaped cell (Table 10-8) with increased mechanical fragility. Some holly-leaf forms can be observed on stained blood smears in addition to the typical sickle shape.

Echinocytes **Echinocytes**, also called *burr cells*, are usually smaller than normal erythrocytes with regular, spinelike projections on their surface (Table 10-8, Figure 10-7c). Their presence is most often artifact in stained blood smears because of the "glass effect" of the slide. The glass releases basic substances that raise the pH of the medium surrounding the cell and induce echinocyte formation. Plasma provides a buffering effect on the cells, and for this reason, blood films made from whole blood may show only certain areas of echinocyte transformation. To determine the in vivo or in vitro nature of echinocytes, a wet preparation can be made in which a drop of blood is enclosed between two plastic cover slips and the unstained individual erythrocytes are observed. If no echinocytes are present in the wet preparation but were noted on the stained blood smears, the cell abnormality occurred as an in vitro artifact.

Echinocytes can appear in blood that has been stored at 4 °C for several days. Consequently, blood specimens from patients receiving transfusions can have echinocytes if blood is taken from the patient immediately after transfusion; however, after a few minutes, the buffering action of patient's plasma causes the transfused echinocyte to resume a normal discoid shape.

For true "in vivo" echinocytes, the characteristic appearance is not related to tonicity of the medium in which the cells are suspended. The shape change is instead thought to result from an increase in the area of the outer leaflet of the lipid bilayer as compared with the inner layer. Echinocyte formation is reversible (i.e., the cell can revert to a discocyte); however, an echinocyte can eventually assume the shape of a spherocyte, presumably because the spleen grooms (removes) the membrane spines; in this circumstance, the cell cannot revert to a normal shape.

Elliptocytes **Elliptocytes**, also called *pencil cells* and *cigar cells*, vary from elongated oval shapes (**ovalocytes**) to elongated rodlike cells (Table 10-8, Figure 10-10 ■). Some laboratory professionals may use the terms elliptocytes and ovalocytes interchangeably, whereas others may use distinct guidelines to delineate the two morphologies. True elliptocytes have parallel sides and a central area of biconcavity with hemoglobin concentrated at both ends (Figure 10-10a). Elliptocytes are formed after the erythrocyte matures and leaves the bone marrow because reticulocytes and young erythrocytes in patients with elliptocytosis are normal in shape. The mechanism of formation is not known but is assumed to involve alterations of the erythrocyte membrane skeleton (Chapter 17). Elliptocytes are the predominant shape of erythrocytes in hereditary elliptocytosis. On the other hand, ovalocytes are fatter on one end than the other and appear to have an egg shape (Figure 10-10b). Ovalocytes are formed in a manner similar to elliptocytes.

Keratocytes **Keratocytes**, also called **helmet cells**, have a concavity on one side and two hornlike protrusions on either end (Table 10-10; Chapter 18). Keratocytes are produced when a fibrin strand impales

an erythrocyte. The two halves of the erythrocyte hang over the strand as saddlebags; the membranes of the touching sides fuse, producing a vacuole-like inclusion on one side. This cell with an eccentric vacuole is called a *blister cell*. The vacuole bursts, leaving a notch with two spicules on the ends.

Knizocytes **Knizocytes** are cells with more than two concavities (Table 10-8). This cell's appearance on stained blood smears can vary depending upon how the cell comes to rest on the flat surface; however, most knizocytes have a dark-staining band across the center with a pale area on either side surrounded by a rim of pink-staining hemoglobin. The mechanism of formation is unknown.

Leptocytes **Leptocytes** are thin, flat cells with normal or larger than normal diameter (Table 10-8). Although the cell's diameter is normal or increased, its volume is usually decreased. The cells have an increased surface-to-volume ratio either as a result of decreased hemoglobin content or increased surface area. The leptocyte is usually cup-shaped like stomatocytes, but the cup has little depth. Target cells can be formed from leptocytes on dried blood smears when the depth of the cup increases.

Schistocytes **Schistocytes** are erythrocyte fragments caused by mechanical damage to the cell (Table 10-8, Figures 10-7b, 10-7c). They appear in a variety of shapes such as triangular, comma, and helmet-shaped. Because schistocytes are fragments of erythrocytes, they are usually microcytic. They maintain normal deformability, but their survival in the peripheral blood is reduced. The fragments can assume a spherical shape and hemolyze or can be removed in the spleen.

Spherocytes **Spherocytes** (Table 10-8, Figure 10-7c) are erythrocytes that have lost their biconcavity because of a decreased surface-to-volume ratio. On stained blood smears, the spherocyte appears as a densely stained sphere lacking a central area of pallor. Although the cell often appears microcytic on stained blood smears, the volume (MCV) is usually normal. The spherocyte is the only erythrocyte that can be called *hyperchromic* because of an increased MCHC.

Stomatocytes In wet preparations, **stomatocytes**, or *mouth cells*, appear as small cup-shaped uniconcave discs (Table 10-8, Figure 10-11 ■). Upon staining, these cells exhibit a slitlike (mouth-like) area of pallor. Normal discocytes can be transformed under certain conditions to stomatocytes and, eventually, to spherostomatocytes. The stomatocyte shape is reversible, but the spherostomatocyte is not. Cationic drugs and low pH cause a gradual loss of biconcavity leading to the stomatocyte and eventually the formation of a sphere. Stomatocytosis is the opposite of echinocytosis; the shape change in stomatocytosis is thought to be the result of an increase in the lipid content or area of the inner leaflet of the membrane lipid bilayer. Stomatocytes also can appear as an artifact on stained blood smears; thus, care should be used in identifying them.

☑ CHECKPOINT 10-6

Review of a peripheral blood smear reveals significant numbers of codocytes and echinocytes. Describe the morphology of these cells. Should you report this on the laboratory report? Why or why not?

Variation in Hemoglobin (Color)

Normal erythrocytes have an MCH of approximately 30 pg. However, the MCHC is a better indicator of chromia or color of erythrocytes on Romanowsky-stained smears. Normally, on stained smears, the erythrocyte has a central area of pallor approximately one-third the diameter of the cell (Figure 10-6a). In certain conditions, RBCs contain less hemoglobin than normal and appear to have a larger than normal central pallor (hypochromia). On the other hand, the only erythrocyte that contains more hemoglobin than normal in relation to its volume is the spherocyte.

Hypochromic Cells **Hypochromic cells** are poorly hemoglobinized erythrocytes with an exaggerated area of central pallor (>1/3 the diameter of the cell) on Romanowsky-stained blood smears. Although occasionally normocytic, hypochromic cells are usually microcytic (Figure 10-12 ■). Hypochromic cells are the result of decreased or impaired hemoglobin synthesis (Table 10-9 ★). When visualizing a blood smear, correlating the automated findings from hematology analyzers to the appearance of cells is important. In the case of hypochromia, the MCHC value will be decreased.

Polychromatophilic Erythrocytes Polychromatophilic erythrocytes (reticulocytes) are usually larger than normal cells with a bluish tinge on Romanowsky-stained blood smears (Figure 10-1). The bluish tinge is caused by the presence of residual RNA in the cytoplasm. Large numbers of these cells are associated with decreased erythrocyte survival or hemorrhage and an erythroid hyperplastic marrow (Table 10-9).

☑ CHECKPOINT 10-7

Increased polychromasia is reported on a blood smear. What is polychromasia, and what other hematologic assay will reflect the presence of polychromasia?

Erythrocyte Inclusions

Erythrocytes do not normally contain any particulate inclusions. When present, inclusions can help direct further investigation because they are associated with certain disease states. Descriptions of

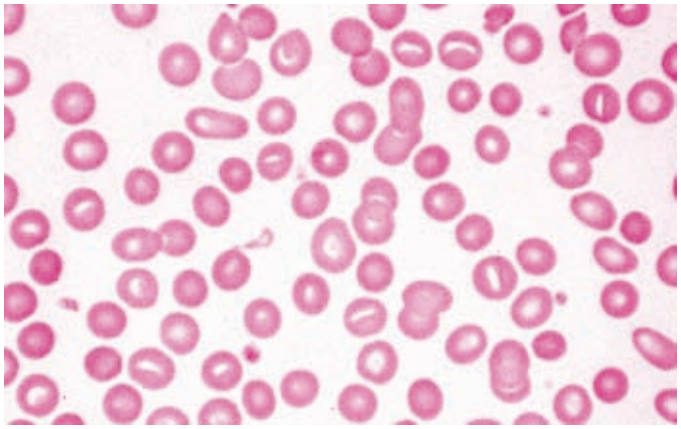


FIGURE 10-11 Stomatocytes. Note the slitlike area of pallor (peripheral blood; Wright-Giemsa stain; 1000× magnification).

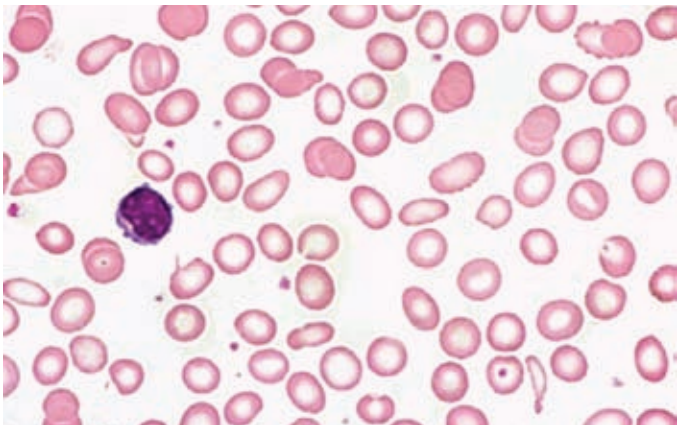


FIGURE 10-12 Microcytic, hypochromic erythrocytes. Compare the size of the erythrocytes with the nucleus of the lymphocyte. Normocytic cells are about the same size as the nucleus. There is only a thin rim of hemoglobin around the periphery of the cells indicating that they are hypochromic (peripheral blood; Wright-Giemsa stain; 1000× magnification).

erythrocyte inclusions as they appear on Romanowsky-stained blood smears, unless otherwise stated, are listed in Table 10-10 ★.

Basophilic Stippling Erythrocytes with **basophilic stippling** are cells with bluish-black granular inclusions distributed across their entire cell area (Table 10-10). The granules can vary in size and distribution from small diffuse to coarse and punctate. The granules, which are composed of aggregated ribosomes, are sometimes associated with mitochondria and siderosomes. Basophilic stippling is not believed to be present in living cells; instead, stippling probably is produced during preparation of the blood smear or during the staining process.[7] Electron microscopy has not shown an intracellular structure similar to basophilic stippling. Cells dried slowly or stained rapidly can demonstrate fine, diffuse stippling as an artifact. Pathologic basophilic stippling is more coarse and punctate.

Cabot Rings **Cabot rings** are reddish-violet erythrocytic inclusions usually occurring in the formation of a figure eight or oval ring (Table 10-10). Cabot rings are thought to be remnants of spindle fibers, which form during mitosis. They occur in severe anemias and in dyserythropoiesis.

Howell-Jolly Bodies **Howell-Jolly bodies** are dark purple or violet spherical granules in the erythrocyte (Table 10-10). These inclusions are nuclear (DNA) fragments usually occurring singly in cells, rarely more than two per cell. Howell-Jolly bodies are associated with nuclear maturation abnormalities. They are thought to occur as a result of an individual chromosome failing to attach to the spindle apparatus during mitosis, and, thus, it is not included in the reformed nucleus. When the nucleus is extruded, the Howell-Jolly body is left behind (until removed by splenic macrophages).

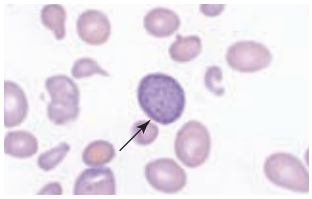
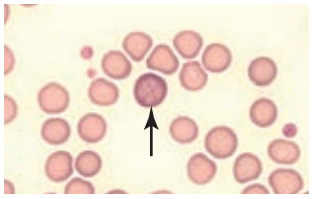
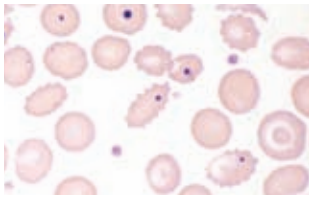
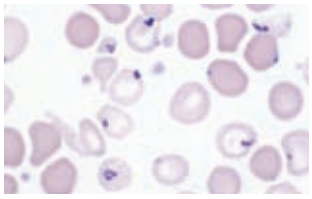
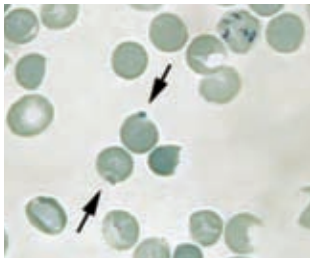
Heinz Bodies **Heinz bodies** *do not* stain with Romanowsky stains but can be visualized with supravital stains or with phase microscopy of the living cell. They appear as 2–3 mcM round masses lying just under or attached to the cell membrane. Heinz bodies are composed of aggregated denatured hemoglobin.

Iron Inclusions Particulate iron molecules can be detected in erythrocytic cells in both normal and abnormal conditions. Intracellular siderotic granules represent iron that has not been

★ **TABLE 10-9** Variations in Erythrocyte Color

Terminology	Description	Associated Physiological or Disease States
Hypochromia	Decreased concentration of hemoglobin in the red cell Red cells have an increased area of central pallor (>1/3 diameter of cell)	May be present in iron deficiency anemia, thalassemia, and other anemias associated with a defect in hemoglobin production (Chapter 12–14)
Polychromasia	Young red cells containing residual RNA Stain a pinkish-gray to pinkish-blue color on Wright's stained blood smears Usually appear slightly larger than mature red cells	Found in increased numbers in hemolytic anemias, newborns, recovery from acute hemorrhage (Chapter 16–20)

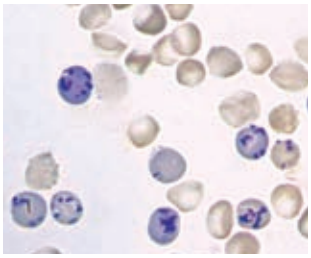
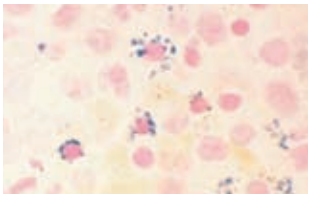
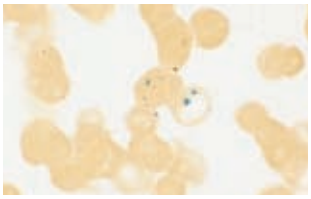
★ TABLE 10-10 Abnormal Erythrocyte Inclusions

Terminology	Description	Associated Disease States	Image
Basophilic stippling	Round or irregularly shaped granules of variable number and size, distributed throughout the RBC Composed of aggregates of ribosomes (RNA) Stain bluish black with Wright's stain	Lead poisoning; anemias associated with abnormal hemoglobin synthesis; thalassemia, Chapters 14, 20	
Cabot rings	Appear as a figure-8, ring, or incomplete ring Thought to be composed of the microtubules of the mitotic spindle Stain reddish violet with Wright's stain	Severe anemias; dyserythropoiesis, Chapter 25	
Howell-Jolly bodies	Small, round bodies composed of DNA usually located eccentrically in the red cell Usually occurs singly, rarely more than two per cell Stains dark purple with Wright's stain	Post splenectomy; megaloblastic anemias; some hemolytic anemias; functional asplenia; severe anemia, Chapter 15	
Pappenheimer bodies	Clusters of granules containing iron that are usually found at the periphery of the cell Visible with Prussian blue stain and Wright's stain	Sideroblastic anemia; thalassemia; other severe anemias, Chapters 12, 14	
Heinz bodies	Bodies composed of denatured or precipitated hemoglobin *Not* visible on Wright's stained blood smears With supravital stain appear as purple, round-shaped bodies of varying size, usually close to the cell membrane Can also be observed with phase microscopy on wet preparations	G6PD deficiency; unstable hemoglobin disorders; oxidizing drugs or toxins; post splenectomy, Chapters 13, 18	

incorporated into hemoglobin. **Sideroblasts** are erythroblasts that contain stainable iron granules whereas **siderocytes** are non-nucleated, mature erythrocytes that contain stainable iron granules (Table 10-11 ★). Sideroblasts and siderocytes can be identified with Perl's Prussian blue iron stain, which stains iron aggregates blue. The granules do not stain with Romanowsky stains. About 20–60% of all erythroblasts in the marrow contain iron that can be visualized with Perl's Prussian blue stain. This number decreases in some pathologic states and can be markedly increased in others. Reticulocytes (Table 10-11) and erythrocytes in the peripheral blood do not normally contain stainable iron aggregates unless the patient has been splenectomized.

Pappenheimer bodies are damaged secondary lysosomes and mitochondria variable in their composition of iron and protein (Table 10-10).[8] This type of inclusion appears as clusters of small granules in erythrocytes and erythroblasts and stains with both Romanowsky and Perl's Prussian blue stains. Romanowsky stains reveal Pappenheimer bodies by staining the protein matrix of the granules whereas Perl's Prussian blue is responsible for staining the iron portion of the granules. Pappenheimer bodies occur only in pathologic states.

★ **TABLE 10-11** Normal Erythrocytic Cell Inclusions

Terminology	Description	Image
Reticulofilamentous substance (reticulocyte)	Artifactual aggregation of ribosomes Not visible on Wright's stained smears; supravital stain (e.g. new methylene blue) must be used Appears as deep blue reticular network	
Sideroblast	Iron granules found in erythroblasts Stains blue with Perl's Prussian blue stain but not with Romanowsky stains	
Siderocyte	Iron granules found in erythrocytes Stains blue with Perl's Prussian blue stain not stain with Romanowsky stains	

☑ CHECKPOINT 10-8

A medical laboratory scientist reports the presence of Howell Jolly bodies in the erythrocytes on a blood smear. What is the composition of these inclusions, and how can he be sure they are not Pappenheimer bodies or Heinz bodies?

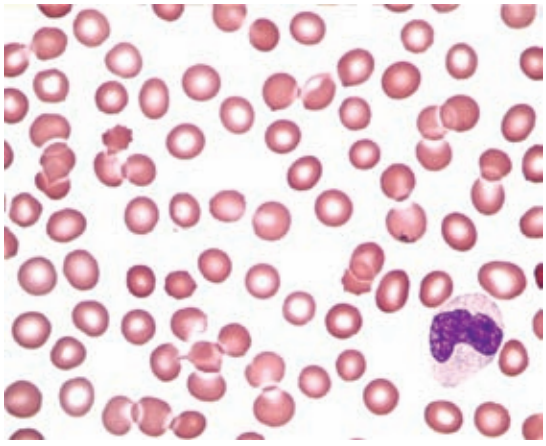
Clinical Laboratory Professional's Review of CBC Data

The role of the laboratory professional is to analyze and interpret the data generated by the automated hematology analyzer and the manual, peripheral blood smear review. The interpretation is essentially a correlation of the various components of the CBC in order to identify the likelihood of abnormal results, pathology, and discrepancies in the generated data. The hematology instrument or laboratory information system produce alerts that include delta checks or that indicate the presence of interfering substances, both of which the laboratory professional must resolve.

Delta checks compare a patient's current clinical values for a test with previous values. This type of quality control can detect sudden changes in a patient's physiology or can be useful in identifying instrument error. Delta checks are particularly important in diagnosis and in monitoring therapy. Abnormal results and the presence of interfering substances (e.g., lipemia, hemolysis) must also be noted and corrected. For a complete list of abnormal test results, use of delta checks, and correction for interfering substances, see Chapter 43, Tables 43-7 and 43-8. In the event that the data can be correlated for diagnosis, the laboratory professional should be able to recommend subsequent testing to the patient's physician.

CASE STUDY (continued from page 165)

The laboratory professional warmed the EDTA blood sample from this patient at 37 °C for 15 minutes and then reanalyzed the CBC on the analyzer and made a new smear. The new smear appeared as in the following figure. The new values were as follows: hemoglobin of 8.2 g/dL (82 g/L), RBC count of 2.63 × 10^{12}/L, and MCV of 91 fL.



8. Calculate the hematocrit, MCH, and MCHC on the warmed specimen. How have they changed?
9. Explain what could have happened in this case.

POST-EXAMINATION PHASE OF THE CBC

After acquisition of the sample, testing, and collecting and reviewing the data, the CBC report is made available to the ordering physician. The steps of the post-examination include reporting the data through a laboratory information system or by other means of physician notification as well as recognition and reporting of critical limits and panic values.

Routine and stat (immediate) orders are generally reported through laboratory information systems; however, some reports can be faxed and paper reports can be used. A reference interval for each parameter of the CBC accompanies each report, as do two identifiers for the patient and the name of the ordering physician. Some reporting systems include flags for abnormal results indicating lower or higher than expected values.

Within the CBC report, the laboratory professional must be keenly aware of the **critical limits** that represent the critical low and high values for a CBC parameter. When values fall above or below the critical limit threshold, they are termed **panic values** and potentially pose a life-threatening risk to the patient. These panic values must be repeated to confirm the result, phoned to the patient's physician, and then properly documented. Each clinical facility determines its critical limits and panic values and the means by which to report those values.

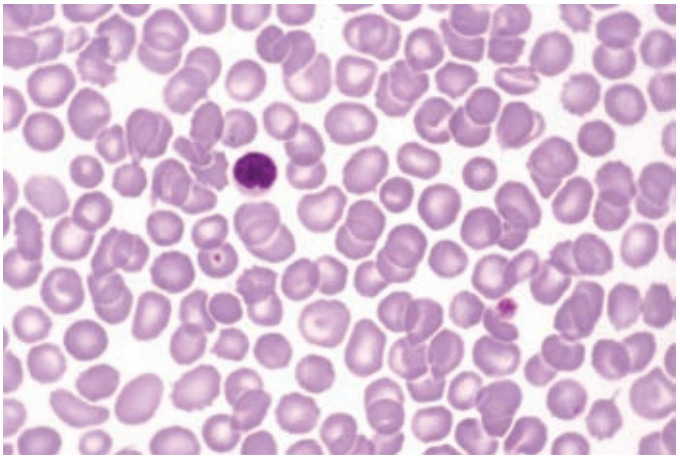
PHYSIOLOGIC VARIATION IN HEMATOLOGIC PARAMETERS

The normal values for hematologic parameters vary depending on age, sex, race, ethnicity, and geographic area. Thus, it is important that this information is available when reviewing data.

CBC Variations in Newborns and Children

Because various parameters of the CBC are dramatically different in newborns compared with adults, the patient's age must be considered when evaluating a patient's blood picture. In premature and term infants, the total WBC count is generally much higher (mean WBC of approximately 25×10^9/L) than adults (Chapter 7). The granulocytes are increased in premature and term infants and drops within the first 72 hours. In the premature infant, immature WBCs may be found in the peripheral circulation for two weeks. Lymphocytes are the predominant cell until age 4. From ages 4–5, the lymphocytes and granulocytes are present in about equal numbers. After age 6, the reference ranges for WBC subsets are similar to those of adults.

The highest normal hemoglobin, hematocrit, and erythrocyte counts are observed at birth. In the neonate, erythrocytes are macrocytic, and the reticulocyte count is 2–6%. During the first week of life, nucleated erythrocytes—as many as 10 per 100 leukocytes—can be present in the peripheral blood. For approximately 2 months after birth, a gradual decrease in the hemoglobin, hematocrit, and erythrocyte counts occurs. The mean MCV of neonates is 108 fL and decreases to a mean of 77 fL between the ages of 6 months and 2 years (Figure 10-13 ■). The mean MCV increases to 80.0 fL by age 5 but does not reach the adult mean of 90 fL until approximately 18 years of age. The MCH changes in parallel to the MCV throughout infancy and childhood, whereas the MCHC remains constant with the adult reference interval.[9] A difference in erythrocyte values between sexes is noted at puberty with females having lower values than males.



■ **FIGURE 10-13** Peripheral blood from a newborn; note the macrocytic erythrocytes (Wright-Giemsa stain; 1000× magnification).

☑ CHECKPOINT 10-9

The following data is found on the CBC of a newborn male.

Hgb = 195 g/L; Hct = 59 L/L;
RBC = 6.50×10^{12}/L; WBC = 17.6×10^9/L

Calculate the MCV and MCHC. Evaluate all of the data for abnormalities.

CBC Variations Between Ethnic Groups and Sexes, in Elderly People, and by Geographic Location

Studies of hematology parameters among different populations have revealed that African Americans have lower hematocrit levels, lower hemoglobin values (about 0.8 g/dL lower), and lower MCVs than Caucasians.[10] Although iron deficiency and the high prevalence of α-thalassemia in African Americans (about 30%), may contribute to the lower hemoglobin and MCV values, the differences narrowed but persisted even when those who tested positive for α-thalassemia and iron deficiency were eliminated from the test group. The WBC count is lower in African Americans primarily due to lower granulocyte counts, but lymphocyte counts are higher.[10]

When comparing hematologic profiles by sex, men show a statistically higher Hb, Hct, RBC, and WBC than women.[11] On the other hand, women have higher platelet counts than men. Differences in the MCV and MCH are minor with slightly lower values in women. Smoking and high alcohol intake have a significant effect on the Hb, Hct, MCV, MCH, and WBC.

Although bone marrow cellularity decreases with age, in the absence of disease, the WBC, hemoglobin, platelet count, and differential are maintained at adult reference ranges in adult populations older than 65.[12] A slight drop in the MCV can occur. The bone marrow appears normal with normal hematopoietic precursor cells.[12,13] Although anemia does not occur due to aging, it becomes more prevalent as the population ages.[13] The most common causes of decreased hemoglobin in elderly people are iron deficiency, Vitamin B_{12} deficiency, anemia of chronic inflammation, kidney disease, and decreased testosterone level. Variations in the CBC are also associated with malignancies that occur at a higher incidence in older adults. Chronic lymphocytic leukemia is the most common leukemia in the Western world and is documented with a mean age of 72.[14] Likewise, the incidence of lymphomas and multiple myeloma is increased in geriatric populations, and myelodysplastic syndromes and acute leukemias have a higher incidence after age 60.[15]

The reference interval for hemoglobin, hematocrit, and RBC should be adjusted upward for those living at high altitudes to account for the normal physiologic response to the lower partial pressure of oxygen.

Summary

The initial hematologic analysis of a patient is called the *complete blood count* (CBC), and the process used to determine it consists of three general phases: pre-examination, examination, and post-examination. The purpose of the CBC pre-examination phase is to ensure the highest quality sample for analysis. Best practices in patient identification, blood collection, and specimen handling should be followed for a seamless transition to the examination phase.

The individual parameters of the CBC are determined by automated and/or manual hematologic analysis in the examination. Whole blood analysis is reported as individual parts consisting of WBCs (enumeration of total WBCs and individual classes), RBCs (enumeration, hemoglobin, hematocrit, indices), and platelets (enumeration and volume). A peripheral blood smear may be prepared for the laboratory professional to evaluate the circulating cells within a patient. Accurate evaluation of the peripheral blood smear can be one of the most difficult skills that the laboratory professional will master because of the numerous variations in WBC, RBC, and platelet morphology.

Finally, the CBC must be interpreted and reported to the requisitioning physician in the post-examination phase. CBC parameters that significantly differ from normal values (either high or low) must be reported with immediacy. The CBC is used to classify hematologic diseases as well as several other pathologies and will be discussed in subsequent chapters.

Review Questions

Level I

1. Which of the following are the correct units for reporting the absolute RBC count using the SI system? (Objective 4)

   A. $\times 10^9$/L

   B. $\times 10^{12}$/L

   C. $\times 10^3$/mcL

   D. $\times 10^9$/fL

2. Which erythrocyte inclusions are composed of DNA and stain blue on Romanowsky stains? (Objective 11)

   A. siderotic granules

   B. Heinz bodies

   C. Howell-Jolly bodies

   D. basophilic stippling

3. Which of the following RBC indices indicates how filled the average RBC is with hemoglobin in terms of weight per unit volume? (Objective 5)

   A. MCV

   B. MCH

   C. MCHC

   D. RDW

4. A blood smear reveals uneven distribution of red blood cells, and the red blood cells appear to be stacked together like a stack of coins. How would you describe this distribution? (Objective 12)

   A. agglutination

   B. rouleaux

   C. anisocytosis

   D. poikilocytosis

5. How would you classify the red cell population with the following indices: MCV 110 fL, MCH 38 pg, MCHC 33 g/dL? (Objective 9)

   A. normocytic, normochromic

   B. macrocytic, normochromic

   C. microcytic, normochromic

   D. microcytic, hypochromic

6. If the cell population in question 5 were homogeneous (absence of anisocytosis), the RDW might show: (Objective 5)

   A. false increase

   B. false decrease

   C. normal reference interval

   D. true increase

7. A peripheral blood smear that has an erythrocyte mixture of macrocytes, microcytes, and normocytes present can best be described as: (Objective 8)

   A. poikilocytosis

   B. polychromatophilia

   C. megaloblastosis

   D. anisocytosis

8. Which of the following erythrocyte inclusions cannot be stained and visualized with Romanowsky stains? (Objective 11)

   A. Pappenheimer bodies

   B. Howell-Jolly bodies

   C. Heinz bodies

   D. basophilic stippling

9. If there is an increase in macrocytic, polychromatophilic erythrocytes on the Romanowsky-stained blood smear, which laboratory test result would correlate with this? (Objective 13)

   A. platelet count

   B. reticulocyte count

   C. leukocyte count

   D. MCHC

10. A routine CBC is to be performed on a blood sample that arrives in the laboratory from an outside clinic 4 hours after it is drawn. The sample is frozen. The sample is: (Objective 3)

    A. acceptable

    B. acceptable but must be warmed before performing the CBC

    C. unacceptable due to improper sample temperature

    D. unacceptable due to improper time restraints

Level II

1. A 53-year-old patient had a hemoglobin of 70 g/L. The reticulocyte count is 15%. Which of the following would you expect on the blood smear? (Objective 4)

   A. poikilocytes

   B. polychromatophilia

   C. agglutination

   D. Howell-Jolly bodies

2. Numerous schistocytes in the patient in question 1 were identified on the blood smear. How could this finding affect the RDW? (Objectives 1, 4)

   A. increase it

   B. decrease it

   C. have no effect

   D. invalidate it

3. Some of the RBCs on a patient's smear contain numerous small blue inclusions. What should be done next to determine what to report about the inclusions? (Objective 5)
   A. perform an iron stain for identification of siderocytes
   B. perform a screen for sickle cell anemia
   C. use new methylene blue stain to confirm an increase in reticulocytes
   D. check the CBC data for an indication of the presence of Howell-Jolly bodies
4. The RDW is found to be 19.5% on a patient. Which of the following should you find increased on the smear? (Objective 1)
   A. macrocytosis
   B. microcytosis
   C. anisocytosis
   D. poikilocytosis
5. Rouleaux is found on a smear of a patient with multiple myeloma. How will this affect the CBC results? (Objective 6)
   A. RBC count will be decreased.
   B. Hematocrit will be increased.
   C. MCHC will be increased.
   D. There will be no effect.
6. A CBC is ordered on a 3-day-old infant with a fever of 100 °F. The laboratory professional notes nucleated RBCs on the peripheral blood smear but is not alarmed by this finding. Why not? (Objective 4)
   A. Nucleated RBCs are a common occurrence during infection.
   B. The fever promoted an increase in RBC production.
   C. Nucleated RBCs are commonly observed in the first 7 days of life.
   D. The nucleated RBCs are likely to be artifacts.
7. A CBC is performed for a patient who has been treated for anemia. The laboratory professional notes an increase in polychromatophilic macrocytes. Which of the following is speculated about the patient from the peripheral blood smear? (Objective 2)
   A. responding to an infection
   B. needs further treatment for the anemia
   C. is responding to the treatment for the anemia
   D. has a decreased RBC count
8. The presence of dacryocytes on a peripheral blood smear is most likely suggestive of which of the following: (Objective 3)
   A. artifact
   B. young RBCs
   C. RBC destruction
   D. splenic removal of RBC inclusions
9. The MCHC result is extremely elevated in a patient's CBC results. Which of the following is a likely cause of this result? (Objective 4)
   A. microcytic RBCs
   B. agglutination of the RBCs
   C. increased hemoglobin and RBC count
   D. high RDW
10. Which of the following poikilocytes are frequently artifacts, not a pathologic finding? (Objective 3)
    A. drepanocytes
    B. echinocytes
    C. spherocytes
    D. schistocytes

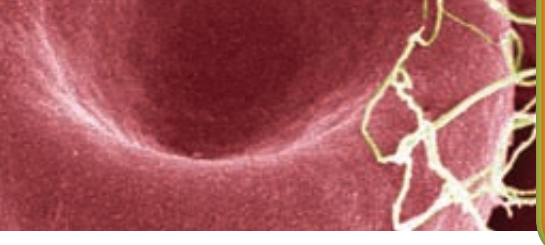
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Hadley GG, Larson NL. Use of sequestrene as an anticoagulant. *Am J Clin Pathol*. 1953;23:613–18.
2. Kennedy JB, Maehara KT, Baker AM. Cell and platelet stability in disodium and tripotassium edta. *Am J Med Technol*. 1981;47:89–93.
3. NCCLS. *Procedures for the handling and processing of blood specimens*. Wayne, PA: NCCLS; 2004. NCCLS document H18-A3.
4. Sennels HP, Jorgensen HL, Hansen AL et al. Diurnal variation of hematology parameters in healthy young males: the Bispebjerg study of diurnal variations. *Scand J Clin Lab Invest*. 2011;71:532–41.
5. Sinha SK, Mandal PK, Mallick J. Pseudothrombocytopenia—a caveat. *J Indian Med Assoc*. 2011;109:476–78.
6. Padayatty J, Grigoropoulos N, Gilligan D et al. Platelet satellitism. *Eur J Haematol*. 2010;84:366.
7. Bessis M. *Living Blood Cells and Their Ultrastructure*, 1st ed. Berlin: Springer-Verlag; 1973: 194–45. The Erythrocytic Series.
8. Glassy ER. *Color Atlas of Hematology: An Illustrated Field Guide Based on Proficiency Testing*. Northfield, IL: CAP; 1998. Updated January 18, 2012.
9. Proytcheva MA. Issues in neonatal cellular analysis. *Am J Clin Pathol*. 2009;131:560–73.
10. Beutler E, West C. Hematologic differences between African Americans and whites: the roles of iron deficiency and α-thalassemia on hemoglobin levels and mean corpuscular volume. 2005. *Blood;*106:740–45.
11. Tsang CW, Lazarus R, Smith W et al. Hematological indices in an older population sample: derivation of healthy reference values. *Clin Chem*. 1998;44:96–101.
12. Ershler WB, Longo DL. Hematology in older persons. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*. 8th ed. New York: McGraw-Hill; 2010 (available at http://www.accessmedicine.com.libproxy.uthscsa.edu/content.aspx?aID=6117071; accessed December 1, 2012).
13. Khier F, Haddad RY. Anemia in the elderly. *Disease-a-Month*. 2010;56:456–67.
14. Friese CR, Earle CC, Magazu LS et al. Timeliness and quality of diagnostic care for medicare recipients with chronic lymphocytic leukemia. *Cancer*. 2011;117:1470–77.
15. Ma X, Does M, Raza A et al. Myelodysplastic syndromes: incidence and survival in the United States. *Cancer*. 2007;109:1536–42.



11 Introduction to Anemia

SHIRLYN B. McKENZIE, PhD
CATHERINE N. OTTO, PhD, MBA

Objectives—Level I

Upon completion of this chapter, the student should be able to:

1. Calculate the reticulocyte production index from reticulocyte results, hematocrit, and RBC count.
2. Identify laboratory tests used to evaluate erythrocyte destruction and production.
3. Given CBC and RPI results, categorize an anemia according to morphologic classification.
4. Correlate polychromatophilia on a blood smear with other laboratory results of erythrocyte production and destruction.
5. List the laboratory tests that can be used to help identify the pathophysiological mechanisms of anemia and provide expected results.
6. Define *hemolysis* and reconcile a normal hemoglobin concentration in compensated hemolytic disease.
7. Assess laboratory results in intravascular and extravascular hemolysis.
8. Summarize the clinical findings associated with anemia.
9. Explain the difference between intrinsic and extrinsic erythrocyte defects.

Objectives—Level II

Upon completion of this chapter, the student should be able to:

1. Relate adaptations to anemia with patient symptoms.
2. Correlate patient history and clinical symptoms with laboratory results in anemia.
3. Evaluate clinical findings of hemolytic anemia and differentiate those associated with acute and chronic disease.
4. Assess bone marrow response to anemia given CBC and reticulocyte results.
5. Assess and interpret bone marrow findings in the presence of anemia.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

6. Compare the sensitivity and specificity as they relate to tests used to screen and confirm a differential diagnosis of anemia.
7. Compare the morphologic and functional classification of anemia.
8. Given laboratory results and clinical findings, classify an anemia in terms of morphology and pathophysiologic mechanism.
9. Compare and contrast the processes of intravascular and extravascular hemolysis and explain how laboratory results can be used to differentiate.
10. Recommend tests that could be necessary to make a diagnosis of hemolytic disease.
11. Choose appropriate reflex tests to determine anemia classification and evaluate the results.
12. Explain the association of anisocytosis and variation in erythrocyte chromia with disease states.

Key Terms

Anemia
CHr
Compensated hemolytic disease
Functional anemia
Functional iron-deficiency anemia
Hemoglobinemia
Hemoglobinuria
Hemolysis
Hemosiderinuria
Immature reticulocyte fraction (IRF)
Megaloblastic
Pancytopenia
Reticulocyte hemoglobin (RET-He)
Reticulocyte production index (RPI)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe and recognize abnormal variation in erythrocyte morphology and distribution on stained smears. (Chapter 10)
- Describe the production, maturation, and destruction of blood cells and explain how the balance between erythrocyte production and destruction is maintained; describe the normal erythrocyte concentration and appearance. (Chapters 3, 5)
- Summarize the role of hemoglobin in gaseous transport. (Chapter 6)
- Discuss the appearance of a normal bone marrow and list reasons that a bone marrow examination could be necessary. (Chapter 38)
- Diagram the process of intravascular and extravascular hemolysis. (Chapter 5)
- Calculate the reticulocyte count (absolute and relative). (Chapter 10)
- Calculate RBC indices and classify erythrocytes based on results. (Chapter 10)
- Discuss the principle and give reference intervals for the following tests: hemoglobin, hematocrit, reticulocyte count, erythrocyte count, and erythrocyte indices; calculate indices. (Chapters 10, 37)

Level II

- Correlate peripheral blood findings with bone marrow appearance. (Chapter 38)
- Correlate CBC results with findings on the peripheral blood smear and other laboratory test results; determine the validity and accuracy of CBC results and suggest corrective action when necessary. (Chapters 10, 37)
- Review the erythrocyte membrane structure. (Chapter 5)

CASE STUDY

We will address this case study throughout the chapter.

George, a 50-year-old male, visited his doctor when he noted that the whites of his eyes appeared yellow and that he had dark urine. His CBC revealed a hemoglobin of 31 g/L (3.1 g/dL).

Given this clinical description, consider what laboratory tests should be ordered to assist in diagnosis.

OVERVIEW

This chapter is a general introduction to anemia. It begins with a description of how anemia develops and the body's adaptations to a decrease in hemoglobin. The emphasis of the chapter is on the laboratory investigation of anemia. This includes discussion of screening tests used to diagnose anemia and other more specific tests used to identify the etiology and pathophysiology of the anemia. Identification of abnormal erythrocyte morphology and its association with anemia is discussed in depth. The chapter concludes with a description of the morphologic and functional classification schemes of anemia and the use of laboratory tests to correctly classify an anemia. The hemolytic anemias are discussed in more depth than the other functional classifications as these anemias have several possible subclassifications that help in understanding the laboratory investigation used to elucidate the pathophysiology.

INTRODUCTION

Anemia is functionally defined as a decrease in the competence of blood to carry oxygen to tissues, thereby causing tissue hypoxia. In clinical medicine, the word refers to a decrease in the normal concentration of hemoglobin and/or erythrocytes. It is one of the most common problems encountered in clinical medicine. However,

anemia is not a disease but the expression of an underlying disorder or disease; it is an important clinical marker of a disorder that could be basic or sometimes more complex. Therefore, once the diagnosis of anemia is made, the physician must determine its exact cause.

Treating anemia without identifying its cause could not only be ineffective but also lead to more serious problems. For example, if a patient experiencing iron-deficiency anemia due to chronic blood loss were given iron or a blood transfusion, the hemoglobin level might temporarily rise; however, if the cause of the deficiency is not isolated and treated, serious complications of the primary disease (cause of blood loss) could develop, and the anemia would probably return after ceasing treatment. Thus, it is necessary to identify and understand the etiology and pathogenesis of an anemia to institute correct treatment.

HOW DOES ANEMIA DEVELOP?

To understand how anemia develops, it is necessary to understand normal erythrocyte kinetics. Total erythrocyte mass (M) in the steady state is equal to the number of new erythrocytes produced per day (P) times the erythrocyte life span (S), which is normally about 100–120 days.

$$\underset{\text{Mass}}{M} = \underset{\text{Production}}{P} \times \underset{\text{Survival}}{S}$$

Thus, the average 70 kg man with 2 liters of erythrocytes must produce 20 mL of new erythrocytes each day to replace the 20 mL normally lost due to cell senescence.[1]

$$\frac{2000\ \text{mL (M)}}{100\ \text{days (S)}} = 20\ \text{mL/day (P)}$$

From this formula, it is clear that if the survival time of the erythrocyte is decreased by one-half, as can occur in hemolysis or hemorrhage, the bone marrow must double production to maintain mass at 2000 mL.

$$\frac{2000\ \text{mL (M)}}{50\ \text{days (S)}} = 40\ \text{mL/day (P)}$$

New erythrocytes are released as reticulocytes. Thus, an increase in the absolute reticulocyte count in the peripheral blood is a result of the increased production of cells. The marrow can compensate for decreased survival in this manner until production is increased to a level 5–8 times normal, which is the maximal functional capacity of the marrow. The increase in erythropoiesis is limited by the amount of iron that can be mobilized for hemoglobin synthesis. The term **functional iron-deficiency anemia** is used when the total body iron is adequate but cannot be mobilized fast enough for the needed increase in erythropoiesis. Thus, if all necessary raw products for cell synthesis are readily available, erythrocyte life span can decrease to about 18 days before marrow compensation is inadequate and anemia develops. If, however, bone marrow production of erythrocytes does not adequately increase when the erythrocyte survival is decreased, the erythrocyte mass cannot be maintained and anemia develops. There is no mechanism for increasing erythrocyte life span to help accommodate an inadequate bone marrow response.

Thus, anemia can develop if (1) erythrocyte loss or destruction exceeds the maximal capacity of bone marrow erythrocyte production or (2) the bone marrow erythrocyte production is impaired. Anemia can be classified according to these principles (functional classification) based on laboratory test results, which aid the physician in diagnosis. The functional classification includes survival defects, proliferation defects, and maturation defects (which have a high degree of ineffective erythropoiesis). A morphologic classification is also possible based on the erythrocyte indices and reticulocyte count. These classifications are discussed later in this chapter.

INTERPRETATION OF ABNORMAL HEMOGLOBIN CONCENTRATIONS

Diagnosis of anemia is usually made after the discovery of a decreased hemoglobin concentration from the CBC results (Chapter 10). Hemoglobin is the carrier protein of oxygen; thus, it is expected that a decrease in its concentration is accompanied by a decrease in oxygen delivery to tissues (**functional anemia**).

Screening for anemia generally relies on the relative hemoglobin concentration and hematocrit (grams of Hb per deciliter of whole blood or liters of RBCs per liter of whole blood). However, the hemoglobin or hematocrit can be misleading as changes in these parameters can reflect altered plasma volume and not a change in the RBC mass.

- In *hypervolemia*, the total blood volume increases. This is primarily caused by a plasma volume increase while the erythrocyte mass remains stable. In this case, the hemoglobin/hematocrit concentration is disproportionately low relative to the red cell mass.
- In *hypovolemia*, such as occurs in dehydration, a decrease in plasma volume relative to the RBC mass occurs. As a result, the hemoglobin/hematocrit can be high or normal relative to the RBC mass.

In acute blood loss, both the plasma volume and RBC mass are decreased, resulting in a normal hemoglobin measurement initially. As the plasma volume increases to re-establish total blood volume for adequate cardiac function, the hemoglobin concentration decreases.

Diagnosis of anemia can require an upward adjustment of hemoglobin and hematocrit reference intervals dependent on the altitude.[2] The hemoglobin reference interval at high altitudes is higher than the reference interval at lower altitudes. Therefore, signs of anemia at high altitudes can occur at higher hemoglobin concentrations than at sea level. Cigarette smoking has a similar effect. The hemoglobin and hematocrit reference interval for cigarette smokers is higher than for nonsmokers.[3]

In most cases, the physician integrates the patient's clinical findings with laboratory test results to correctly diagnose the illness. The examples mentioned serve to emphasize the fact that when making a diagnosis of anemia, the physician depends not only on laboratory test results but also considers patient history, physical examination, and symptoms.

ADAPTATIONS TO ANEMIA

Signs and symptoms of anemia range from slight fatigue or barely noticeable physiologic changes to life-threatening reactions depending on:

- Rate of onset
- Severity of blood loss
- Ability of the body to adapt

With rapid loss of blood as occurs in acute hemorrhage, clinical manifestations are related to hypovolemia and vary with the amount of blood lost. A normal person can lose up to 1000 mL, or 20%, of total blood volume and not exhibit clinical signs of the loss at rest, but tachycardia is common with mild exercise.[4] Severe blood loss of 1500–2000 mL or 30–40% of total blood volume leads to circulatory collapse and shock. Death is imminent if the acute loss reaches 50% of total blood volume (2500 mL).

Slowly developing anemias can show an equally severe drop in hemoglobin as is seen in acute blood loss, but the threat of shock or death is not usually present. The reason for this apparent discrepancy is that in slowly developing anemias, the body has several adaptive mechanisms that allow organs to function at hemoglobin levels of up to 50% less than normal. The adaptive mechanisms are of two types: an increase in the oxygenated blood flow to the tissues and an increase in oxygen utilization by the tissues (Table 11-1 ★).

Increase in Oxygenated Blood Flow

Increasing the cardiac rate, cardiac output, and circulation rate can increase oxygenated blood flow to the tissues. Oxygen uptake in the alveoli of the lungs is increased by deepening the amount of inspiration and increasing the respiration rate. In anemia, decreased blood viscosity due to the decrease in erythrocytes and decrease in peripheral resistance help to increase the circulation rate, delivering oxygen to tissues at an increased rate. Blood flow to the vital organs, the heart and brain, can preferentially increase whereas flow to tissues with low oxygen requirements and normally high blood supply such as skin and the kidneys decreases.

Increase in Oxygen Utilization by Tissue

An important compensatory mechanism at the cellular level that allows the tissue to extract more oxygen from hemoglobin involves an increase in 2,3-BPG (2,3-bisphosphoglycerate—also known as *2,3-diphosphoglycerate/2,3-DPG*) within the erythrocytes (Chapter 6). An increase in erythrocyte 2,3-BPG permits the tissues to extract more oxygen from the blood even though the PO_2 remains constant; this shifts the oxygen dissociation curve to the right. It is not clear exactly how anemia stimulates this increase in cellular 2,3-BPG.

Another adaptive mechanism at the cellular level involves the Bohr effect. The scarcity of oxygen causes anaerobic glycolysis by muscles and other tissue, which produces a buildup of lactic acid. In addition, H^+ is generated from carbonic acid (H_2CO_3) formed during the transport of CO_2 from the tissues to the lungs (Chapter 6). This acidosis decreases hemoglobin's affinity for oxygen in the capillaries, thus causing release of more oxygen to the tissues and shifting the oxygen dissociation curve to the right.

Even with these physiologic adaptations, different anemic patients respond differently to similar changes in hemoglobin levels. The extent of the physiologic adaptations is influenced by:

1. Severity of the anemia
2. Competency of the cardiovascular and respiratory systems
3. Oxygen requirements of the individual (physical and metabolic activity)
4. Duration of the anemia
5. Disease or condition that caused the anemia
6. Presence and severity of coexisting disease

★ **TABLE 11-1** Adaptations to Anemia

Increase in oxygenated blood flow
• Increase in respiration rate and deepen inspiration • Increase in cardiac rate • Increase in cardiac output • Increase in circulation rate • Preferential increase in blood flow to vital organs
Increase in oxygen utilization by tissues
• Increase in 2,3-BPG in erythrocytes • Decreased oxygen affinity of hemoglobin in tissues due to Bohr effect

DIAGNOSIS OF ANEMIA

Anemia can impair an individual's ability to carry on activities of daily living and decrease the individual's quality of life. Thus, accurate diagnosis and treatment are essential to improve patient outcomes. The diagnosis of anemia and determination of its cause are made by using a combination of information received from the patient history, the physical examination, and the laboratory investigation (Table 11-2 ★).

History

The patient's history including symptoms can reveal some important clues as to the cause of the anemia. Information solicited by the physician should include dietary habits, medications taken, possible exposure to chemicals or toxins, and description and duration of the symptoms. The most common complaint is tiredness. Muscle weakness and fatigue develop when there is not enough oxygen available to burn fuel for the production of energy.

Severe drops in hemoglobin can lead to a variety of additional symptoms. When oxygen to the brain is decreased, headache, vertigo, and syncope can occur. Dyspnea and palpitations from exertion, or occasionally while at rest, are not uncommon complaints. The patient should be questioned as to any overt signs of blood loss, such as hematuria, hematemesis, and bloody or black stools. Studies of anemia in the elderly reveal that in this population, anemia is associated with increased cognitive impairment and falls as well as a decline in physical performance.[5] Family history can help define the rarer hereditary types of hematologic disorders. For example, sickle cell anemia and thalassemia are frequently manifested to some degree in several members of the immediate family.

★ **TABLE 11-2** Important Information for Evaluating a Patient for Anemia

Diagnosis of anemia and determination of its cause requires information obtained from the patient history, physical examination, and laboratory data.

I. Patient history
  A. Dietary habits
  B. Medications
  C. Exposure to chemicals and toxins
  D. Symptoms and their duration
    1. Fatigue
    2. Muscle weakness
    3. Headache
    4. Vertigo
    5. Syncope
    6. Dyspnea
    7. Palpitations
    8. Dark or red urine
  E. Previous record of abnormal blood examination
  F. Family history of abnormal blood examination

II. Signs of anemia obtained by physical examination
  A. Skin pallor
  B. Pale conjunctiva
  C. Koilonychia
  D. Hypotension
  E. Jaundice
  F. Smooth tongue
  G. Neurological dysfunction
  H. Hepatomegaly
  I. Splenomegaly
  J. Bone deformities in congenital anemias
  K. Gallstones
  L. Extramedullary hematopoietic masses

III. Laboratory investigation
  A. Erythrocyte count
  B. Hemoglobin
  C. Hematocrit
  D. Erythrocyte indices: MCV, MCH, MCHC
  E. Reticulocyte count, reticulocyte production index (RPI), corrected reticulocyte count, CHr or Ret-He, IRF
  F. Blood smear examination
  G. Leukocyte and platelet quantitative and qualitative examination
  H. Peripheral blood smear evaluation for presence of spherocytes, schistocytes and other poikilocytes, and erythrocyte inclusions
  I. Tests to measure erythrocyte destruction depending on other information available: serum bilirubin, haptoglobin, hemopexin, lactate dehydrogenase (LD), methemalbumin, urine hemosiderin, fecal and urine urobilinogen, blood in urine, expired CO
  J. Bone marrow examination (depending upon results of other laboratory tests and patient clinical data)

Physical Examination

Physical examination of the patient helps the physician detect the adverse effects of a long-standing anemia (Table 11-2). Signs of anemia are associated with decreased hemoglobin levels and in hemolytic anemias with increased hemoglobin catabolism and erythropoiesis. General physical findings include the following:

- Changes in epithelial tissue from oxygen deprivation are noted in some patients. Skin pallor is easily noted in most Caucasian patients, but because of variability in natural skin tone, pale conjunctiva is a more reliable indicator of anemia. The presence of pallor, particularly conjunctival pallor, has been shown to be a cost-effective and feasible method to screen for anemia in a variety of settings.[6,7]
- Hypotension can accompany significant decreases in blood volume.
- Heart abnormalities can occur as a result of the increased cardiac workload associated with the physiologic adaptations to anemia. Cardiac problems usually occur only with chronic or severe anemia (hemoglobin <7 g/dL).
- Organomegaly of the spleen and liver are of primary importance in establishing the extent of involvement of the hematopoietic system in the production and destruction of erythrocytes. Massive splenomegaly is characteristic of some hereditary chronic anemias. Splenic hypertrophy is a constant finding in hemolytic anemias with extravascular hemolysis such as in some autoimmune hemolytic anemias when the spleen is the primary site of destruction of antibody-sensitized erythrocytes.
- Expansion of the bone marrow, consequently thinning cortical bone and widening the spaces between inner and outer tables of bone, is present in chronic severe hemolytic anemias. In children, this expansion is evident as skeletal abnormalities. These bone changes can result in spontaneous fractures and *osteoarthropathy*.[8]
- Anemia can occur secondarily to a defect in hemostasis. The presence of bruises, ecchymoses, and petechiae indicates that the platelets may be involved in the disorder that is producing the anemia.

In addition to these general physical findings associated with anemia, findings can be associated with a particular type of anemia. These include koilonychia in iron deficiency and a smooth tongue in megaloblastic anemia. Hemolytic anemias are associated with jaundice and dark or red urine (if intravascular hemolysis is present). Gallstones consisting primarily of bilirubin are common in congenital and other chronic hemolytic anemias. Extramedullary hematopoietic masses can be found in the hereditary hemoglobinopathies, some of which are thought to be extrusions of the marrow cavity through thinned bone cortex. Small colonies of erythrocytes also can be found in the spleen, liver, lymph nodes, and perinephric tissue. These masses can cause pressure symptoms on adjacent organs.[8]

In addition to determining the extent of anemic manifestations, physical examination helps to establish the underlying disease process causing the anemia. Some disorders associated with anemia include chronic diseases such as rheumatoid arthritis as well as malignancies, gastrointestinal lesions, kidney disease, parasitic infection, and liver dysfunction. Anemia in pregnancy is common. The anemia can be due to a variety of underlying conditions including iron or folate deficiency, inflammatory conditions, and hemodilution.[9]

Laboratory Investigation

After the physical examination and patient history, a health care provider who suspects the patient has anemia orders laboratory tests (Table 11-2). Initially, screening tests are performed to determine whether anemia is present and to evaluate erythrocyte production and destruction/loss. The initial screening test is the complete blood count (CBC), which includes red blood cell (RBC) count, hemoglobin, hematocrit, RBC indices, white blood cell (WBC) count, platelet count, and, depending on instrumentation, the differential count (Chapter 10). Depending on these test results, additional tests such as the reticulocyte count, bilirubin, and microscopic review of the blood smear for abnormal cell morphology can be suggested. In addition, the urine and stool can be examined for the presence of blood. When combined with the information from the history and physical examination of the patient, results of these tests can give insight to the cause of the anemia. These routine tests can be followed by a protocol of specific diagnostic tests that help establish the etiology and pathophysiology of the anemia. These specific tests will be discussed in the appropriate chapters on anemia.

Erythrocyte Count, Hematocrit, and Hemoglobin

The erythrocyte count, hematocrit, and hemoglobin are determined to screen for the presence of anemia. In a clinic or physician's office with limited resources, screening may be limited to either the hematocrit or hemoglobin. If one of these screening tests is abnormal, it is helpful to calculate the red cell indices.

A decreased concentration in one or more of these parameters, based on the individual's age and sex, should be followed by other laboratory tests to help establish criteria for diagnosis. The Centers for Disease Control and Prevention (CDC) recommended cutoff values for a diagnosis of anemia according to age and sex are provided in Table 11-3 ★. Upward adjustments for these cutoff values should be utilized for individuals living at high altitudes and for those who smoke. There is a direct dose–response relationship between the amount smoked and the hemoglobin level.[3] The CDC recommended adjustments are included in Tables 11-4 ★ and 11-5 ★. Hemoglobin and hematocrit values also vary in pregnancy with a gradual decrease in the first two trimesters and a rise during the third trimester (Table 11-6 ★).

★ **TABLE 11-3** Hemoglobin (Hb) and Hematocrit (Hct) Cutoffs for a Diagnosis of Anemia in Children, Nonpregnant Females, and Males

Age (yrs) by Sex	Hb (g/dL)	Hct (%)
Both sexes		
1–1.9	11.0	33.0
2–4.9	11.2	34.0
5–7.9	11.4	34.5
8–11.9	11.6	35.0
Female		
12–14.9	11.8	35.5
15–17.9	12.0	36.0
≥18	12.0	36.0
Male		
12–14.9	12.3	37.0
15–17.9	12.6	38.0
≥18	13.6	41.0

Based on fifth percentile values from the Second National Health and Nutrition Examination survey conducted after excluding persons with a higher likelihood of iron deficiency.
Centers for Disease Control (CDC). CDC criteria for anemia in children and childbearing-aged women. *MMWR Morb Mortal Wkly Rep*. 1989;38(22):400–4.

★ **TABLE 11-4** Altitude Adjustments for Hemoglobin (Hb) and Hematocrit (Hct) Cutoffs for a Diagnosis of Anemia

Altitude (ft)	Hb (g/dL)	Hct (%)
<3000	—	—
3000–3999	+0.2	+0.5
4000–4999	+0.3	+1.0
5000–5999	+0.5	+1.5
6000–6999	+0.7	+2.0
7000–7999	+1.0	+3.0
8000–8999	+1.3	+4.0
9000–9999	+1.6	+5.0
>10,000	+2.0	+6.0

Centers for Disease Control (CDC). CDC criteria for anemia in children and childbearing-aged women. *MMWR Morb Mortal Wkly Rep*. 1989;38(22):400–4.

Although anemia in elderly persons is prevalent, it should not be considered a normal part of aging. The third National Health and Nutrition Examination Survey (NHANES III, 1988–94) studied a group of noninstitutionalized older individuals and found that after age 65, the prevalence of anemia rose to 11% in men and 10.2% in women.[10,11] The prevalence for those in nursing homes is higher. The highest prevalence of anemia is in those over 85 years of age (26% of men and 20% of women). In this group, one-third was due to blood loss/nutritional deficiency, one-third was due to anemia of chronic disease, inflammation, or chronic renal failure, and one-third was unexplained. The unexplained anemia can be due to multiple causes. Even when allowing for a difference in reference interval, the prevalence of anemia in African Americans over 65 years of age is three times higher than in Caucasians. Prevalence in Mexican Americans is similar to that in Caucasians.

Variations in hemoglobin also are reported to occur as a result of blood-drawing techniques. Hemoglobin values are about 0.7 g/dL

★ **TABLE 11-5** Adjustments for Hemoglobin (Hb) and Hematocrit (Hct) Cutoffs for a Diagnosis of Anemia in Smokers

Characteristic	Hb (g/dL)	Hct (%)
Nonsmoker	—	—
Smoker (all)	+0.3	+1.0
½–1 pack/day	+0.3	+1.0
1–2 packs/day	+0.5	+1.5
>2 packs/day	+0.7	+2.0

Centers for Disease Control (CDC). CDC criteria for anemia in children and childbearing-aged women. *MMWR Morb Mortal Wkly Rep*. 1989;38(22):400–4.

★ **TABLE 11-6** Hemoglobin Cutoffs for a Diagnosis of Anemia in Pregnancy by Month and Trimester[a]

	Gestation (wks)/Trimester							
	12/1†	16/2	20/2†	24/2	28/3	32/3†	36/3	40/Term
Mean Hb (g/dL)	12.2	11.8	11.6	11.6	11.8	12.1	12.5	12.9
5th percentile Hb values (g/dL)	11.0	10.6	10.5	10.5	10.7	11.0	11.4	11.9
Equivalent 5th percentile Hct values (%)†	33.0	32.0	32.0	32.0	32.0	33.0	34.0	36.0

[a] Based on pooled data from four European surveys of healthy women taking iron supplements. Hb values adapted for the trimester-specific cutoffs
†Hematocrit
From Centers for Disease Control (CDC). CDC criteria for anemia in children and childbearing-aged women. *MMWR Morb Mortal Wkly Rep*. 1989;38(22):400–4.

higher if the patient's blood is obtained while the individual is in an upright position rather than supine. Prolonged vasoconstriction by the tourniquet can cause hemoconcentration of the sample and elevate the hemoglobin value.

Erythrocyte Indices

Because abnormal morphology is characteristic of distinct types of anemia, the erythrocyte indices (MCV, MCH, MCHC, RDW) give important clues as to the pathophysiology of the anemia and thus help to direct reflex testing (Chapter 10, 37). For instance, microcytic hypochromic cells are highly suggestive of iron-deficiency anemia, whereas macrocytic normochromic cells are associated with vitamin B_{12} or folate deficiency. The indices are used in the morphologic classification of anemia.

CASE STUDY (continued from page 179)

George's only complaint was dark urine and the yellow color of his eyes. His CBC results were hemoglobin 31 g/L (3.1 g/dL), hematocrit 0.08 L/L (8%), RBC count 0.71×10^{12}/L, RDW 21.6, reticulocyte count 22%. Calculate the erythrocyte indices.

1. Does this information suggest acute or chronic blood loss? What is the significance of the RDW?

☑ CHECKPOINT 11-1

Explain why a 30-year-old female who smokes a pack of cigarettes a day and lives in the Rocky Mountains can be diagnosed with anemia when her hemoglobin is 12 g/dL.

Reticulocyte Count

The peripheral blood reticulocyte count indicates the degree of effective bone marrow erythropoietic activity and is one of the most useful and cost-effective laboratory tests in monitoring anemia and response to therapy (Chapters 10, 37). It is also helpful in directing the initial investigation of anemia that assists in classification of anemia. Increased numbers of polychromatophilic erythrocytes on Romanowsky-stained smears indicates an increased reticulocyte count.

The reticulocyte count is commonly performed on an automated hematology instrument using a variety of methodologies including fluorescent flow cytometry or scattered light with methylene blue staining, depending on the manufacturer. These automated instruments provide a higher degree of accuracy, precision, and standardization than the manual reticulocyte counts. The availability of automated hematology instruments that can perform reticulocyte counts has significantly decreased the frequency in which a manual reticulocyte method is used[12] (Chapter 39). In both automated and manual methods, reticulocyte test results are expressed as a percentage of reticulocytes in relation to total RBC count or as the absolute number. The reference interval varies among laboratories and the procedure used, but it is about 0.5–2.0% or 25–75 $\times$ 10^9/L for manual reticulocyte counts. Because of a lack of standardization, there is no single reference range for reticulocyte parameters determined by flow cytometry.[13] Laboratories need to determine reference intervals for their own instrument and method.

In healthy, aged individuals, the lifespan of the erythrocyte appears to decrease.[14] This is compensated for by an active bone marrow so that the hemoglobin and hematocrit remain in the reference interval of other adults. There is, however, a slight reticulocytosis, reflecting the increased production of erythrocytes.

The corrected reticulocyte count is a means to adjust the reticulocyte count in proportion to the severity of anemia. In this procedure, the percentage of reticulocytes is multiplied by the ratio of the patient's hematocrit to an average age- and sex-appropriate normal hematocrit:

$$\frac{\text{Patient hematocrit}}{\text{Normal hematocrit}} \times \%\ \text{reticulocyte} = \text{Corrected reticulocyte count (\%)}$$

For practical purposes, the corrected reticulocyte count or preferably absolute reticulocyte count is used to assess the degree of erythropoiesis in anemic patients.[12] In patients with anemia, a corrected reticulocyte count $<2\%$ or an absolute reticulocyte count $<75 \times 10^9$/L is associated with hypoproliferative anemias, whereas a corrected count $>2\%$ or an absolute count $>100 \times 10^9$/L is associated with an appropriate response to blood loss and hemolytic anemias.[15] Counts between 75

☑ CHECKPOINT 11-2

Is it possible to have an increased relative reticulocyte count but an absolute reticulocyte count in the reference range? Explain.

and 100 × 10^9/L should be interpreted while considering factors such as the severity of the anemia and other clinical information.

Quantitation of Reticulocyte Immaturity

Under normal physiologic conditions when there is no anemia, the reticulocytes are released into the peripheral blood where they spend another day maturing to the erythrocyte. When the need for erythrocytes in the circulation increases, the bone marrow releases reticulocytes earlier than normal. These more immature reticulocytes are called *stress reticulocytes* or *shift reticulocytes*. They appear as large polychromatophilic cells on the Romanowsky-stained blood smear. It takes longer for these reticulocytes to mature in the peripheral blood because the bone marrow maturation time is added to the peripheral blood maturation time (Table 11-7 ★). The more severe the anemia, the earlier the reticulocyte is released. In a stimulated marrow, hematocrit levels of 35%, 25%, and 15% (0.35, 0.25, 0.15 L/L) are associated with early reticulocyte release and a prolongation of the reticulocyte maturation in peripheral blood to approximately 1.5, 2.0, and 2.5 days, respectively. This is similar to the left shift in granulocytes seen in peripheral blood when the need for granulocytes is increased. To correct for the prolongation of maturation of these circulating shift reticulocytes and anemia, the **reticulocyte production index (RPI)** is calculated by using the following formula:

$$\frac{\text{Patient's hematocrit}}{\text{0.45 L/L (normal hematocrit)}} \times \frac{\text{Reticulocyte count (\%)}}{\text{Reticulocyte maturation time (days)}} = \text{RPI}$$

For example, if a patient with a 0.25 L/L hematocrit had a 15% reticulocyte count, the RPI would be:

$$\frac{0.25\ \text{L/L}}{0.45\ \text{L/L}} \times \frac{15\%}{2.0} = 4.2\ \text{RPI}$$

The RPI is a good indicator of the adequacy of the bone marrow response in anemia. Generally speaking, an RPI >2 indicates an appropriate bone marrow response, whereas an RPI <2 indicates an inadequate compensatory bone marrow response (hypoproliferation) or an ineffective bone marrow response. When utilized in this way, the reticulocyte count provides a direction for the course of investigation concerning anemia etiology and pathophysiology.

An advantage of the automated reticulocyte count is that the corrected reticulocyte count, absolute reticulocyte count, and RPI calculations do not need to be performed. Most automated hematology instruments not only provide a relative and absolute reticulocyte count but also assess reticulocytes for maturity level. Reticulocyte maturity level can be classified based on semiquantitative assessment of RNA concentration within the maturing erythrocyte. Younger reticulocytes contain more RNA than more mature reticulocytes. The methods vary and include measurement of fluorescence, absorbance, or light scatter of stained cells. The term used for this index is the **immature reticulocyte fraction (IRF)** to reflect the least mature fraction of reticulocytes. Reference intervals vary by manufacturer.[16]

The IRF can be helpful in evaluating bone marrow erythropoietic response to anemia, monitoring anemia, and evaluating response to therapy. In anemia, an increased IRF generally indicates an adequate erythropoietic response, whereas a normal or subnormal IRF reflects an inadequate or no response to the anemia.[17] As the bone marrow increases production of erythrocytes, an observable increase in the IRF occurs *before* an increase in the reticulocyte count or an increase in hemoglobin, hematocrit, or RBC count. After bone marrow transplantation, an increased IRF has been observed as one of the first signs of cell recovery. In patients receiving human recombinant erythropoietin (rHuEPO) or iron therapy for anemia, an increased IRF can indicate increased erythropoietic activity or a response to the rHuEPO.

The Clinical Laboratory Standards Institute (CLSI) recommends that the IRF index replace the RPI.[12] Laboratories that do not have instruments that measure this parameter can use the RPI.

Another component of automated reticulocyte analysis is the reticulocyte hemoglobin parameter. Reticulocyte hemoglobin is a measure of the hemoglobin content of reticulocytes, which reflects the availability of functional iron for the cell and the incorporation of iron into hemoglobin over the last several days. Thus, reticulocyte hemoglobin indicates response or lack of response to iron therapy. The Advia 120 and Bayer 2120 by Siemens-Healthcare directly measure the hemoglobin content of reticulocytes, reported as **CHr** (analogous to the MCH of erythrocytes) as well as the mean reticulocyte cell hemoglobin concentration (CHCMr; analogous to the MCH and MCHC of erythrocytes). The Sysmex Automated Hematology Analyzers that perform reticulocyte counts have an equivalent CHr parameter called the **reticulocyte hemoglobin (RET-H*e*)**, which is measured using the forward scatter and side fluorescence of the reticulocytes and a proprietary algorithm. A recent study found that the normal ranges for RBC and reticulocyte hemoglobin in picograms for the two methods are comparable.[18]

★ **TABLE 11-7** Correlation of the Hematocrit with Reticulocyte Maturation Time in the Peripheral Blood

Hematocrit (L/L)	Reticulocyte Maturation Time (days)
≥0.35	1.0
≥0.25–0.35	1.5
≥0.15–0.25	2.0
≤0.15	2.5

CASE STUDY *(continued from page 184)*

George's RBC count is 0.7 × 10^{12}/L, and his reticulocyte count is 22%.

2. Calculate his absolute reticulocyte count. Is this count increased, decreased, or normal?

Blood Smear Examination

Although doing so is not always necessary, reviewing the stained blood smear assists in diagnosing the type of anemia in about 25% of the cases.[19] Various pathological conditions intrinsic or extrinsic to the cell can alter the erythrocyte's normal morphology. Careful examination of a stained blood smear reveals these morphologic aberrations. (Description of specific types of poikilocytes and disorders with which they are associated are included in Chapter 10.) Some shapes and sizes are particularly

★ **TABLE 11-8** Diseases Associated with Variation and Abnormalities in Erythrocyte Size

Terminology	Associated Disease States
Anisocytosis	Anemias associated with an increased RDW (see Table 11-14)
Microcytosis (MCV <80 fL)	Iron-deficiency anemia; thalassemia; sideroblastic anemia, long-standing anemia of chronic disease
Macrocytosis (MCV >100 fL)	Megaloblastic anemias; hemolytic anemia with reticulocytosis; recovery from acute hemorrhage; liver disease; asplenia; aplastic anemia; myelodysplasia; endocrinopathies; alcoholism

characteristic of specific underlying hematologic disorders or malignancies (Table 10-8). Abnormal shapes to report include schistocytes, dacryocytes, spherocytes, acanthocytes, and marked erythrocyte shape abnormalities in normocytic anemia without evidence of hemolysis. If artifactual morphology is suspected, the erythrocytes should be examined in a wet preparation. If the abnormal morphology is present in this preparation, the possibility of artifacts can be eliminated (Chapter 10).

Characterization of predominant erythrocyte size and variation in size is helpful in morphologic classification of anemia. As with poikilocytes, anisocytes are sometimes associated with particular pathologic conditions (Table 11-8 ★). Anisocytosis can be detected by examining the blood smear and/or by reviewing the MCV, histograms, cytograms, and RDW.

Microcytes (MCV <80 fL) are usually hypochromic (decreased MCHC and MCH) but can be normochromic or hyperchromic if spherocytes are present (Figure 11-1 ■). Microcytes are usually associated with anemias characterized by defective hemoglobin formation (Table 11-8).

Macrocytes (MCV >100 fL) usually contain an adequate amount of hemoglobin (normal MCHC and normal to increased MCH) (Figure 11-2 ■). These cells are associated with impaired DNA synthesis as occurs in Vitamin B_{12} or folate deficiency as well as other diseases (Table 11-8). Anemias associated with reticulocytosis, such as hemolytic anemias, can have an increased MCV as young reticulocytes are generally larger than mature erythrocytes.

CASE STUDY *(continued from page 185)*

George's blood smear revealed marked spherocytosis.

3. Explain the importance of this finding.
4. Explain George's abnormal indices.

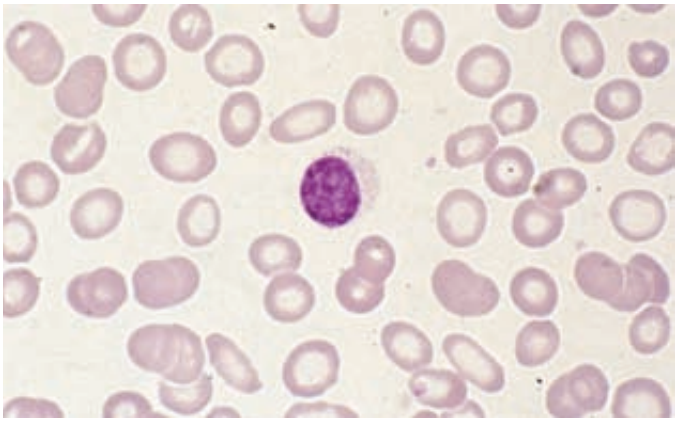
Other abnormalities found on stained blood smears that can assist in diagnosis and classification of anemia include variations in erythrocyte color (Table 10-9), presence of erythrocyte inclusions (Table 10-10), and abnormal distribution of erythrocytes (Table 10-6).

Leukocyte and Platelet Abnormalities

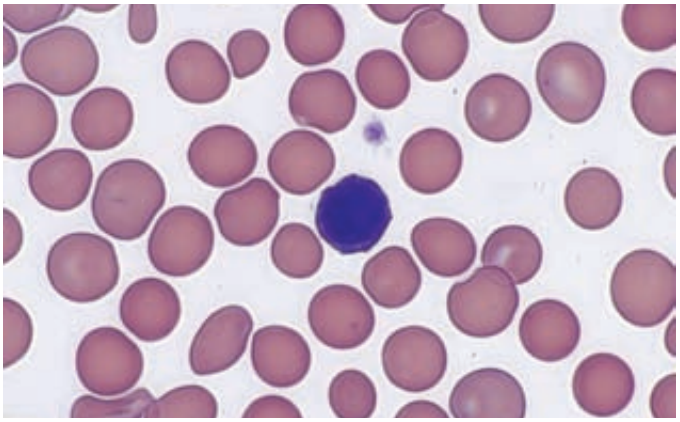
Some nutritional deficiencies, stem cell disorders, and bone marrow abnormalities affect the production, function, and/or morphology of all hematopoietic cells; thus, evaluation of the quantity and morphology of leukocytes and platelets can supply additional important data as to the cause of anemia.

Tests for Erythrocyte Destruction

Tests of erythrocyte destruction are important in evaluating erythrocyte survival (Table 11-9 ★). If the hemoglobin concentration is stable over at least several days in an anemic patient, the



■ **FIGURE 11-1** Microcytic, hypochromic erythrocytes. Compare the size of the erythrocytes with the nucleus of the lymphocyte. Normocytic cells are about the same size as the nucleus. There is only a thin rim of hemoglobin around the periphery of the red blood cells, indicating they are hypochromic. Note the elliptocytes (peripheral blood; Wright-Giemsa stain; 1000× magnification).



■ **FIGURE 11-2** Macrocytic erythrocytes. Compare the size of the erythrocytes with the nucleus of the lymphocyte. Spherocytes are present (peripheral blood; Wright-Giemsa stain; 1000× magnification).

★ **TABLE 11-9** Common Laboratory Findings Reflecting Increased/Decreased Production and Destruction of Erythrocytes

Increased Bone Marrow Production of Erythrocytes	Increased Erythrocyte Destruction	Decreased Bone Marrow Production of Erythrocytes
Reticulocytosis (>100 × 10^9/L; RPI >2)	Anemia	Anemia
Increased IRF	Presence of spherocytes, schistocytes, and/or other poikilocytes	Decreased reticulocytes (<25 × 10^9/L); RPI <2; corrected reticulocyte count <2%; decreased IRF
Leukocytosis	Positive direct antihuman globulin test (DAT)	Erythroid hypoplasia in the bone marrow; increased M:E ratio
Nucleated erythrocytes in the peripheral blood	Decreased haptoglobin and hemopexin	
Polychromasia of erythrocytes on Romanowsky-stained blood smears	Decreased glycosylated hemoglobin	
Normoblastic erythroid hyperplasia in the bone marrow	Increased fecal and urine urobilinogen	
	Increased bilirubin (unconjugated)	
	Hemoglobinemia[a]	
	Hemoglobinuria[a]	
	Hemosiderinuria[a]	
	Methemoglobinemia[a]	
	Increased serum LD	
	Increased expired CO	

[a]Associated only with intravascular hemolysis
IRF, immature reticulocyte fraction; LD, lactic dehydrogenase; RPI, reticulocyte production index; CO, carbon monoxide

measurements of erythrocyte production including marrow cellularity and RPI are indirect measurements of erythrocyte destruction. Serum unconjugated bilirubin is primarily derived from hemoglobin catabolism; its concentration in the absence of hepatobiliary disease can yield further information concerning erythrokinetics. Increased unconjugated bilirubin indicates increased hemoglobin catabolism, either intravascular or extravascular. Conversely, anemias that are due to chronic and acute blood loss and hypoproliferative anemias are associated with normal or decreased serum bilirubin because the number of erythrocytes catabolized is decreased. Cytoplasmic maturation abnormalities can also be accompanied by normal to decreased serum bilirubin even though erythrocyte destruction is increased. This happens because insufficient heme is being synthesized (hypochromic cells), and less heme is being catabolized. Thus, the bilirubin level should always be interpreted together with the degree and type of anemia. It has been suggested that too many variables affect serum bilirubin levels to make it a reliable measurement of RBC destruction.

Other laboratory tests can be used to evaluate erythrocyte turnover or blood loss (Table 11-9). Hemosiderin in urine (**hemosiderinuria**), decreased plasma haptoglobin and hemopexin (as a result of increased consumption), and increased methemalbumin are associated with increased intravascular hemolysis. Certain biochemical constituents that are concentrated in blood cells are released to the peripheral blood as the cell lyses, and these constituents indicate the degree of cellular destruction. In anemias associated with ineffective erythropoiesis or hemolysis, these biochemical constituents will be increased in the blood. The most commonly measured constituents include uric acid, the main end product of purine metabolism, and lactate dehydrogenase (LD), an enzyme that is present in the cell cytoplasm.

Bone Marrow

Bone marrow evaluation usually is not necessary to determine the cause of an anemia. However, it can provide supplemental diagnostic information in anemic patients when other laboratory tests are not conclusive. For example, bone marrow evaluation in hypoproliferative anemias can reveal myelodysplasia or infiltration of the marrow with malignant cells or granulomas. Erythroid hyperplasia of the bone marrow with decreased amounts of fat is more pronounced in hemolytic anemia than in any of the nonhemolytic anemias. Consequently, the myeloid-to-erythroid ratio (M:E) is decreased (reference interval is 1.5 to 3.3; mean 2.3).

Erythrocyte Survival Studies

Erythrocyte survival studies are helpful in defining a hemolytic process in which erythrocyte survival is only mildly decreased. In mild hemolysis, laboratory findings typical of extravascular or intravascular hemolysis can be absent. Survival studies give insight into the rate and mechanism of hemolysis. To study erythrocyte survival, a sample of the patient's blood is removed and labeled in vitro with trace amounts of radionuclide. The most common label for erythrocytes, and that recommended by the International Committee for Standardization in Hematology, is radioactive chromium (^{51}Cr). The chromium penetrates the erythrocytes and remains trapped there. This labeled sample is injected intravenously into the patient. To determine the erythrocyte survival pattern, small samples of the patient's blood are assayed at specific time intervals for radioactivity levels. The erythrocyte life span is expressed as the time it takes for blood radioactivity to decrease by one-half ($T½^{51}$Cr) starting 24 hours after injection. About 1% of the ^{51}Cr is normally eluted from surviving cells daily. In addition, only 1% of the labeled cells can be expected to have a life span of 100–120 days because only 1% of the total erythrocyte mass is replaced each day.

The remaining labeled cells have expected life spans from 0–100 days. Taking these facts into consideration, the normal T½ with this method has been determined to be 25–32 days. A steady state is necessary for accurate interpretation of erythrocyte survival studies because blood loss or transfusions can alter the data significantly. Labeled erythrocytes in this method are also useful in determining the sites of erythrocyte destruction. The amount of radioactivity taken up by an organ can be measured by scanning the body for ^{51}Cr deposition and is proportional to the number of erythrocytes destroyed there.

Differential Diagnosis of Anemia Based on Laboratory Tests

The choice of laboratory tests for the differential diagnosis of anemia should depend on the test's specificity and sensitivity. A highly sensitive test will likely be positive when the disorder is present. A highly specific test will be negative when the disorder is not present. Highly sensitive tests are good for screening for the disorder, and highly specific tests are good for confirming the diagnosis of the disorder. Laboratory professionals should keep this in mind when creating algorithms for anemia testing. Operating characteristics for some tests used in diagnosing anemia can be found in the literature.

☑ CHECKPOINT 11-3

What laboratory test is the least invasive and most cost effective to evaluate erythrocyte production in the presence of anemia?

CLASSIFICATION OF ANEMIAS

The purpose of the classification of anemias is to assist the physician in identifying the cause by using laboratory test results in addition to other clinical data. The classification also is useful to laboratory professionals when they correlate various test results for accuracy and make suggestions for additional reflex testing. Although specific diagnosis is the ultimate goal of any anemia classification system, it must be kept in mind that anemia frequently develops from more than one mechanism, complicating correlation and interpretation of laboratory test results. In addition, complicating factors can alter the typical findings of a specific anemia. For example, pre-existing iron deficiency can inhibit the reticulocytosis that normally accompanies acute blood loss or mask the macrocytic features of folic acid deficiency. In these cases, laboratory test results can depend on which mechanism predominates. Anemias can be classified by either morphology (morphologic classification) or pathophysiology (functional classification).

Morphologic Classification

Anemias can be initially classified morphologically according to the average size and hemoglobin concentration of the erythrocytes as indicated by the erythrocyte indices (Figure 11-3 ■). This morphologic classification is helpful because MCV, MCH, and MCHC are determined when anemia is diagnosed, and certain causes of anemia are characteristically associated with specific erythrocyte size (large, small, or normal) and hemoglobin content (normal or abnormal). The general categories of a morphologic classification include macrocytic, normochromic; normocytic, normochromic; and microcytic, hypochromic.

It must be stressed that, although an anemia initially seems to belong in one of these categories, the morphologic expression can be the result of a combination of factors. For example, a combined deficiency of iron and folate can result in a normal MCV even though iron deficiency is normally microcytic and folate deficiency is normally macrocytic. These complicated cases usually can be detected by examining the blood smear for specifics of erythrocyte morphology.

A morphologic assessment of anemia, however, is not sufficient; determining the etiology of anemia through additional laboratory tests beginning with the IRF or RPI, and corrected reticulocyte count yields even more meaningful information. Serum iron studies are most helpful in identifying the pathophysiology of microcytic anemias because the IRF or RPI is variable in these cases. Patient history and physical examination are essential for a differential diagnosis within given classifications.

Microcytic, Hypochromic Anemia

Microcytic, hypochromic anemias are associated with defective hemoglobin synthesis. Serum iron studies and occasionally hemoglobin electrophoresis are usually adequate to differentiate the causes of these anemias (Chapters 12, 14).

Macrocytic Anemia

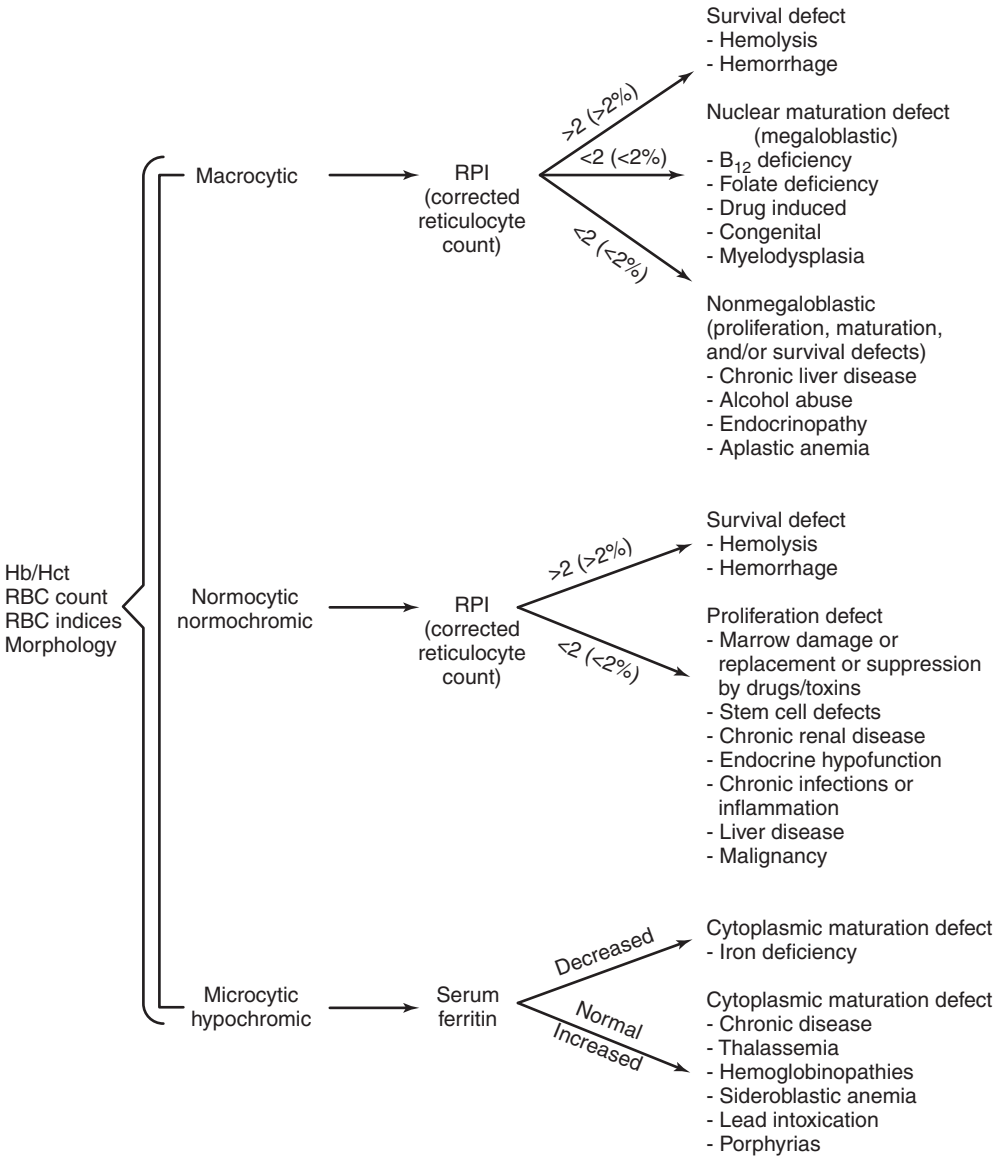
Macrocytic anemias are associated with hemolytic anemias (RPI >2), nuclear maturation defects (megaloblastic anemia, RPI <2), or nonmegaloblastic anemia (RPI <2). Diagnostic features of megaloblastic anemia such as hypersegmented granulocytes can be found on peripheral blood smears and supported by low vitamin B_{12} or folic acid levels (Chapter 15). Hemolytic anemias with an increased MCV due to reticulocytosis can usually be diagnosed by using other laboratory tests and reviewing the blood smear. Nonmegaloblastic macrocytic anemias have various causes, but clinical symptoms, history, and other laboratory tests are usually sufficient to arrive at a diagnosis.

Normocytic, Normochromic Anemia

Many anemias have normal RBC morphology. These include hypoproliferative anemias characterized by an RPI <2 and survival defects with a RPI >2. The hypoproliferative anemias are characterized by a hypocellular bone marrow with normal or increased M:E ratio. Hemolytic anemias have a hypercellular bone marrow with decreased M:E ratios.

Functional Classification

Because the normal bone marrow compensatory response to decreased peripheral blood hemoglobin levels is an increase in erythrocyte production, persistent anemia can be expected as the result of three pathophysiologic mechanisms: (1) a proliferation defect (decreased production), (2) a maturation defect, or (3) a survival



■ **FIGURE 11-3** Classification of anemias using function and morphology reflexed by RPI and/or serum ferritin. (Corrected reticulocyte count is in parentheses.)

defect (increased destruction). These are considered to be the three functional classifications of anemia (Figure 11-4). The functional classification uses the absolute reticulocyte count, corrected reticulocyte count, IRF or RPI, and/or serum iron studies to categorize an anemia. Proliferation and maturation defects usually have a normal or decreased IRF and/or RPI <2 and corrected reticulocyte count $<2\%$; survival defects are characterized by an increased IRF and/or RPI >2 and corrected reticulocyte count $>2\%$. Some anemias can be grouped into more than one functional subgroup (e.g., thalassemia) because they have characteristics of each (i.e., maturation and survival defects).

Although some anemias can be the result of several mechanisms, one mechanism is usually dominant. The initial step in approaching an anemic patient is the identification of this dominant mechanism. If the functional and morphologic classifications of anemia are combined, the result is a classification using the reticulocyte count, iron studies, and morphology of the erythrocyte (Figure 11-4 ■). If an anemia does not fit into any of these categories, it is probably multifactorial.

Proliferation Defects

Proliferation defects are characterized by decreased proliferation, maturation, and release rates of erythrocytes in response to anemia (Figure 11-4). The most characteristic laboratory findings of proliferation defects are normocytic, normochromic erythrocytes, decreased absolute reticulocyte count, decreased corrected reticulocyte count and IRF, and RPI <2, signifying a marrow output of reticulocytes inadequate for the degree of anemia. Serum bilirubin levels are normal or decreased because of the decrease in cell production. The bone marrow is hypocellular with normal or increased iron stores. Decreased proliferation can be caused by inappropriate erythropoietin production or production of cytokines that inhibit erythropoiesis. This trophic basis is responsible for the anemias

associated with malignancies, chronic renal disease, chronic inflammation,[20] and certain endocrinopathies.

Conversely, erythropoietic-stimulating mechanisms can be normal, but the bone marrow can fail to respond to the stimulus appropriately. This failure can occur when the bone marrow is infiltrated with fibrous, neoplastic, or granulomatous tissue or when chemicals, drugs, or radiation have damaged the marrow. Differentiating these causes of hypoproliferation is possible by observing whether all cell lineages are affected or only the erythrocytes are involved. If the proliferation defect is due to inappropriate erythropoietin production, decreased proliferation is limited to the erythrocytic lineage. In contrast, marrow damage or infiltration is characterized by hypoplasia of all normal hematopoietic cells in the bone marrow, producing **pancytopenia** (a decrease in all blood cells) in the peripheral blood. In addition, poikilocytosis and a leukoerythroblastic peripheral blood picture, presumably caused by damage of the normal sinusoidal barrier, usually accompany marrow infiltration.

Many proliferative defects also are associated with decreased erythrocyte lifespan; however, survival is only moderately decreased and could easily be compensated for by a normally functioning marrow or a normal cytokine stimulus. Hypoproliferation is rarely caused by an abnormality in the hematopoietic stem cells.

Maturation Defects

Maturation defects disrupt the orderly process of either nuclear or cytoplasmic development producing qualitatively abnormal cells (Figure 11-4). The erythrocytes are macrocytic in nuclear defects and microcytic in cytoplasmic defects. Despite the abnormal maturation process, the marrow attempts to increase production of erythrocytes, resulting in bone marrow erythroid hyperplasia. However, because these cells are often intrinsically abnormal, many are destroyed before they can be released to the peripheral blood (ineffective erythropoiesis). Because many of the abnormal erythrocytes are not released to the peripheral blood, the corrected reticulocyte count, the absolute

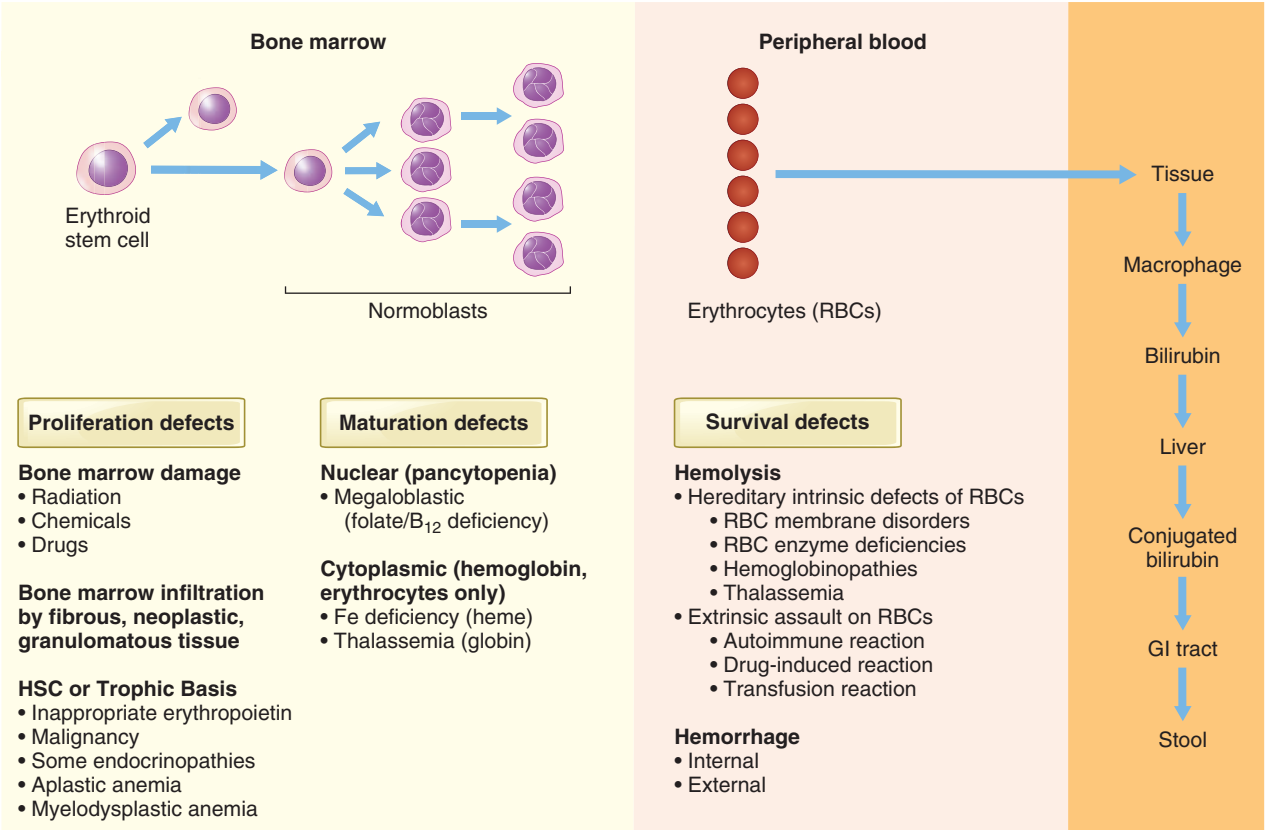


FIGURE 11-4 Functional classification of anemia. Anemia can be due to proliferation, maturation, and/or survival defects. Proliferation and maturation defects are due to defective erythropoiesis in the bone marrow. Survival defects are caused by increased destruction of erythrocytes. This destruction can occur in the bone marrow if the cells are intrinsically abnormal and/or in the peripheral circulation. Destruction of cells in the bone marrow is called *ineffective erythropoiesis*. In some cases, an anemia can be due to several causes such as in thalassemia. Although thalassemia is classified as a maturation defect, it also is characterized by ineffective erythropoiesis in which the abnormal cells are destroyed before they reach the peripheral blood. Thus, there also is a survival defect.

reticulocyte concentration, and IRF are decreased and the RPI <2. Poikilocytes indicative of abnormal erythropoiesis are frequently present in direct proportion to the severity of the anemia.

Abnormal hemoglobin production causes cytoplasmic maturation defects. Therefore, the defect is limited to the erythroid lineage. Hemoglobin production can be impaired due to one or more of the following: limited iron supply, defective iron utilization, decreased globin synthesis, and defective porphyrin (heme) synthesis. Most erythrocytes produced in association with cytoplasmic maturation defects are microcytic and hypochromic with a variable degree of poikilocytosis. These anemias are best differentiated using iron studies (Chapter 12).

Because all developing hematopoietic cells have nuclei, nuclear maturation defects affect all hematopoietic cell lineages and probably other body cells as well. As a result, the peripheral blood can reflect not only anemia but also pancytopenia with characteristic morphologic changes apparent in all cell lineages. The distinctive morphologic changes in cells are collectively termed **megaloblastic** (delayed nuclear development in comparison to cytoplasmic development).

Survival Defects

Survival defects are the result of premature loss of circulating erythrocytes either by hemorrhage or hemolysis (Figure 11-4). **Hemolysis** is the premature destruction of erythrocytes. In this type of defect, bone marrow proliferation increases and maturation is orderly. The absolute reticulocyte concentration and IRF are increased, the corrected reticulocyte count is >2%, and the RPI is typically >2. The blood film reflects this increased erythropoietic activity by the presence of polychromatophilic macrocytes. If the bone marrow is able to compensate for the decreased erythrocyte life span by increasing production at the same rate as the cells are lost or hemolyzed, anemia does not develop. This condition is referred to as **compensated hemolytic disease**, which can rapidly develop into anemia if (1) erythrocyte destruction accelerates beyond the compensatory capacity of the bone marrow (hemolytic crises) or (2) the marrow suddenly stops producing erythrocytes (aplastic crises).

Hemolytic anemia can be further classified based on the cause of the shortened erythrocyte survival, either intrinsic or extrinsic to the erythrocyte (Table 11-10 ★). *Intrinsic* refers to hereditary abnormalities of the erythrocyte itself, whereas *extrinsic* refers to an antagonist in the red cell's environment that causes injury to the erythrocyte. Other possible classifications of hemolytic anemias include mode of onset, site of hemolysis, and predominant poikilocyte present in peripheral blood (Table 11-10). Many anemias, although not primarily hemolytic, have a hemolytic component. These include the hemoglobinopathies, thalassemia, iron-deficiency anemia, and megaloblastic anemia.

In contrast to poikilocytes that are formed in the bone marrow as a result of dyserythropoiesis typical of proliferation and maturation defects, poikilocytes of a survival defect are formed after the cell leaves the marrow. The most common poikilocytes are schistocytes and spherocytes. The schistocyte is the result of intravascular mechanical trauma to the cell, such as a shearing by fibrin strands or damage by passing through abnormal capillaries. Spherocytes indicate extravascular erythrocyte membrane damage because of membrane loss to phagocytes in the spleen. Generally, the erythrocyte population is normocytic and normochromic. It is possible, however, that macrocytosis can prevail, depending on the degree of reticulocytosis, or that microcytosis can predominate, depending on the number of schistocytes or microspherocytes.

Sites of Destruction in Hemolytic Anemia

Hemolysis can occur within the circulation (intravascular) or within the macrophages of the spleen, liver, or bone marrow (extravascular). In some cases, depending on the degree of damage to the cell, destruction occurs both intravascularly and extravascularly. The results of laboratory tests can provide important clues to the hemolytic process.[21]

Intravascular hemolysis can be caused by (1) the activation of complement on the erythrocyte's membrane, (2) physical or mechanical trauma to the erythrocyte, or (3) the presence of soluble toxic substances in the erythrocyte's environment (Table 11-11 ★).

When the erythrocyte is hemolyzed intravascularly, free hemoglobin is released into the plasma and binds to haptoglobin, and the haptoglobin–hemoglobin complex is taken to the liver to be catabolized (Chapter 5). When haptoglobin is depleted, hemopexin (another plasma protein) complexes with heme and takes it to the liver to be catabolized. Hemopexin is quickly depleted when the complex is cleared faster than the liver can synthesize hemopexin. A decrease in hemopexin is secondary to a reduction in haptoglobin.

Laboratory findings of intravascular hemolysis include **hemoglobinemia**, **hemoglobinuria**, hemosiderinuria, methemoglobinemia, decreased haptoglobin, and decreased hemopexin. Serum lactic dehydrogenase (LD) can increase to as much as 800 IU/L (upper limit of reference interval is 207 IU/L). LD can be an early and sensitive indicator of intravascular hemolysis because the erythrocyte releases LD into the plasma in intravascular hemolysis, and it is cleared from the plasma more slowly than hemoglobin.[22]

Extravascular hemolysis is more common than intravascular hemolysis. Extravascular hemolysis that results in premature erythrocyte destruction occurs when phagocytes in the tissues remove erythrocytes from circulation. Hemoglobin is not released directly to the plasma, so there is no hemoglobinemia, hemoglobinuria, or hemosiderinuria. Hemoglobin is degraded within the phagocyte to heme,

★ **TABLE 11-10** Possible Classifications of Hemolytic Anemia Based on Pathophysiology, Etiology, and/or Laboratory Findings

Source of Defect	Mode of Onset	Site of Hemolysis	Predominant Poikilocyte
Intrinsic to red cell (intracorpuscular)	Inherited	Extravascular	Spherocyte
Extrinsic to red cell (extracorpuscular)	Acquired	Extravascular or intravascular depending on extent of cell damage	Schistocyte

★ **TABLE 11-11** Anemias Characterized by Intravascular Hemolysis

Activation of Complement on the Erythrocyte Membrane	Physical or Mechanical Trauma to the Erythrocyte	Toxic Microenvironment of the Erythrocyte
Paroxysmal nocturnal hemoglobinuria	Microangiopathic hemolytic anemia	Bacterial infections
Paroxysmal cold hemoglobinuria	Abnormalities of the heart and great vessels	*Plasmodium falciparum* infection
Some transfusion reactions	Disseminated intravascular coagulation	Venoms
Some autoimmune hemolytic anemias		Arsine poisoning
		Acute drug reaction in G6PD deficiency
		Intravenous administration of distilled water
		Thermal injury

G6PD = glucose-6-phosphate dehydrogenase

iron, and globin. (See Chapter 6 for a description of the process of the breakdown of the hemoglobin molecule.)

Laboratory findings in hemolytic anemias associated with extravascular hemolysis are measurements of the products of heme catabolism. These findings include increases in expired carbon monoxide, carboxyhemoglobin, serum bilirubin (especially the unconjugated fraction), and both urine and fecal urobilinogen. In severe or chronic extravascular hemolysis, haptoglobin and hemopexin levels also can be decreased.

Antibodies directed against the erythrocyte commonly cause hemolytic anemia associated with extravascular hemolysis. Antibody and complement attached to the cell membrane make the erythrocyte a target for removal from the circulation by phagocytes. The antihuman globulin (AHG) test (or direct antiglobulin test, DAT) is helpful in identifying erythrocytes sensitized with antibodies and/or complement.

Bone marrow macrophages are responsible for the removal of maturing precursor cells that are intrinsically abnormal (ineffective erythropoiesis). Many hemolytic anemias that are associated with inherited defects of the erythrocyte membrane, hemoglobin, and intracellular enzymes have some degree of ineffective erythropoiesis. Although in most cases many of the abnormal cells never enter the peripheral blood, a significant number do gain access to the peripheral blood. These cells are not physiologically equipped to withstand the assaults of the peripheral circulation and are damaged. The hepatic or splenic macrophages then remove the damaged cells (Table 11-12 ★).

★ **TABLE 11-12** Anemias Characterized by Extravascular Hemolysis

Origin	Anemias
Inherited erythrocyte defects	Thalassemia
	Hemoglobinopathies
	Enzyme deficiencies
	Membrane disorders
Acquired erythrocyte defects	Megaloblastic anemia
	Spur cell anemia
	Vitamin E deficiency in newborns
Immunohemolytic anemias	Autoimmune
	Drug induced
	Some transfusion reactions

Source of Defect in Hemolytic Anemias

Hemolytic anemias can be classified as intrinsic or extrinsic according to the cause of the shortened erythrocyte survival (Table 11-13 ★). Refer to the earlier explanation of *intrinsic* and *extrinsic*.

Intrinsic Defects. With few exceptions, intrinsic defects are hereditary. The site of hemolysis in intrinsic defects is usually extravascular. In some cases, intrinsic defects render the cell more susceptible than normal cells to damage by environmental (extracorpuscular) factors. Although extracorpuscular factors can be involved, the initiating event in hemolysis is considered to be the intrinsic erythrocyte abnormality. These intrinsic abnormalities include:

- Structural defects of the erythrocyte membrane that can cause the membrane to become abnormally permeable, rigid, or unstable and easily fragmented (Chapters 5, 17)
- Structurally abnormal hemoglobins that result in hemoglobin insolubility or instability (Chapter 13)
- Deficiencies of erythrocyte enzymes necessary for maintaining hemoglobin and membrane sulfhydryl groups in the reduced state or for maintaining adequate levels of adenosine triphosphate (ATP) for cation exchange (Chapter 18)

Extrinsic Defects. Extrinsic defects are usually acquired, and hemolysis can be either intravascular or extravascular. The erythrocytes, as innocent bystanders, are damaged by chemical, mechanical, or physical agents (Chapters 19, 20). Substances in the circulation can be toxic to the cell and cause direct cell hemolysis or alter the cell membrane, leading to removal of the cell in the spleen. Trauma to the erythrocyte in the circulation can cause the cell to fragment, producing striking abnormalities on the blood smear. Immune-mediated destruction can occur when antibodies and/or complement attach to the erythrocytes resulting in their removal by macrophages in the spleen or liver.

Classification Using the Red Cell Distribution Width

Changes in the MCV and RDW are relatively reproducible in certain anemias. When used with the reticulocyte count, the differential diagnosis is narrowed and classification of the anemia is facilitated.[23] It has been suggested that the classification of anemias use the terms *heterogeneous* (increased RDW) and *homogeneous* (normal RDW)

★ **TABLE 11-13** Classification of Hemolytic Anemias Based on Underlying Defect

Classification	Underlying Defect	Examples
Intrinsic (inherited)	Membrane defects	Hereditary spherocytosis
		Hereditary elliptocytosis
		Hereditary pyropoikilocytosis
		Hereditary stomatocytosis
		Dehydrated hereditary stomatocytosis
		Paroxysmal nocturnal hemoglobinuria (acquired)
	Enzyme disorders	Glycolytic pathway enzyme deficiencies
		Hexose-monophosphate shunt enzyme deficiencies
	Abnormal hemoglobins	Thalassemia
		Structural hemoglobin variants (e.g., sickle cell anemia)
Extrinsic (acquired)	Antagonistic plasma factors	Chemicals, drugs
		Animal venoms
		Infectious agents
		Plasma lipid abnormalities
		Intracellular parasites
		Splenomegaly
	Traumatic physical cell injury	Microcirculation lesions
		Thermal injury
		March hemoglobinuria
	Immune-mediated cell destruction	Autoimmune
		Alloimmune
		Drug induced

in conjunction with the descriptive morphologic terms *microcytic*, *normocytic*, and *macrocytic* (e.g., homogeneous macrocytic, heterogeneous macrocytic) (Table 11-14 ★).

Studies of anemic individuals provided the following information regarding the relation between categories of anemia and RDW.[22,23]

1. Hypoproliferative anemias have a normal RDW regardless of the MCV.
2. Maturational defect anemias (excluding the rare hereditary types) have an increased RDW regardless of the MCV or the degree of anemia. The RDW is increased in these individuals before anemia develops or before abnormal cells can be identified on the smear.
3. The RDW is normal after acute hemorrhage if iron supplies are adequate.
4. Uncompensated hemolytic anemias have a high RDW, whereas compensated hemolytic states have a normal RDW.

☑ CHECKPOINT 11-4

Explain why classification of anemia is important, and give the categories of the morphologic and functional classifications.

Laboratory Testing Schemas for Anemia Diagnosis

Knowledge of the functional and morphologic classification of anemias is necessary to design a cost-effective laboratory testing approach that aids in specific diagnosis. Only appropriate tests that help identify the cause of anemia should be performed on the patient's laboratory workup. Guidelines within ICD codes for reimbursement of laboratory tests by third-party payers make it clear that test ordering must be rationally based. Web Figure 11-1 shows general schemas of laboratory testing that are useful in diagnosing anemias. It should be remembered that the physician always takes the patient's clinical history and performs the physical examination before beginning a laboratory workup. The information gained in these ways can eliminate the need for some tests and/or suggest additional tests. These schemas will gain more meaning as you read the following chapters on each group of anemias.

CASE STUDY *(continued from page 186)*

5. Classify George's anemia morphologically and functionally.

★ **TABLE 11-14** Classification of Anemias by MCV and RDW

	Normal RDW (homogeneous)	Increased RDW (heterogeneous)
Normocytic	Acute hemorrhage	Immune hemolytic anemia
	Splenic pooling	Early iron, B_{12}, or folate deficiency
	Chronic disease	Dimorphic anemia (e.g., folate and iron deficiency)
	Chronic leukemia	Sideroblastic anemia
	Renal disease	Myelofibrosis
		Sickle cell anemia/trait
		Chronic liver disease
		Myelodysplastic syndrome
Microcytic	Heterozygous thalassemia	Iron deficiency
	Chronic disease	Homozygous thalassemia
	Hemoglobin E trait	Hb S/B^{thal}
		Hb H disease
		Hemolytic anemia with schistocytes
Macrocytic	Chronic liver disease	Immune hemolytic anemia with marked reticulocytosis
	Aplastic anemia	B_{12} or folate deficiency
	Chemotherapy	CLL with high lymph count
	Alcohol ingestion	Cytotoxic chemotherapy
	Antiviral medications	Chronic liver disease
		Myelodysplastic syndrome

Summary

Anemia is a decrease in the competence of blood to carry adequate amounts of oxygen to the tissue resulting from an insufficient concentration of hemoglobin. Diagnosis of anemia is made with a combination of information from the patient history, physical examination, and laboratory investigation. Initially, routine laboratory tests are performed to determine the presence of anemia and to evaluate erythrocyte production and destruction. These tests can include erythrocyte count, hemoglobin, hematocrit, erythrocyte indices, reticulocyte count, and blood smear examination. More specific tests can be performed based on the results of these routine tests.

The erythrocyte indices can be used to determine the size and hemoglobin content of erythrocytes. Because some anemias are characterized by specific erythrocyte morphology, the indices are helpful in initially classifying the anemia.

The manual reticulocyte count is routinely reported in relative terms: the number of reticulocytes per erythrocytes expressed in percent. Automated reticulocyte counts are reported in both relative and absolute terms. More information is available from the absolute count, corrected reticulocyte count, IRF, RPI, and CHr (RET-He). Generally, the reticulocyte count in an anemic patient should be increased ($>100 \times 10^9$/L) if the bone marrow is increasing production of erythrocytes. Absolute counts $<25 \times 10^9$/L in anemic patients is indicative of a hypoproliferative response.

Examination of the blood film is helpful in assessing anisocytosis and poikilocytosis. *Anisocytosis* is a variation in erythrocyte size and is calculated and expressed as the red cell distribution width (RDW) on some automated cell counters. It is not uncommon to find a variety of cell sizes in some anemias. Poikilocytosis is a variation in cell shape. Specific shapes give clues to the cause of anemia.

Bone marrow examination is indicated if laboratory tests give inconclusive results. Bone marrow is examined for cellularity, cellular structure, M:E ratio, and iron stores.

Anemias are generally classified by a functional or morphologic scheme or by a combination of the two. The morphologic classification includes three general categories based on erythrocyte indices: normocytic, normochromic; macrocytic, normochromic; and microcytic, hypochromic. The functional classification uses the IRF or RPI and serum iron studies to classify the anemias according to pathophysiology: proliferation defect, maturation defect, and survival defect. Each of these classifications has subclassifications. Some anemias such as thalassemia can fall into more than one functional classification group because they have characteristics of both (i.e., hemolytic and maturation). These classifications help the laboratory professional and physician design a cost-effective approach to laboratory testing to reach a specific diagnosis.

Review Questions

Level I

1. Which of the following is characteristic of severe intravascular hemolysis? (Objective 7)
   A. decreased bilirubin
   B. increased hemopexin
   C. decreased urobilinogen
   D. decreased haptoglobin
2. A patient with anemia has an RPI of 2.3 with an MCV of 103 fL. How would you classify this anemia? (Objective 3)
   A. macrocytic
   B. normocytic
   C. microcytic
   D. maturation defect
3. Which of the following tests will give information about rate of erythrocyte production? (Objective 2)
   A. RPI
   B. serum bilirubin
   C. serum ferritin
   D. MCV
4. A patient has the following results: RBC count, 2.5×10^{12}/L and hemoglobin 5.3 g/dL, hematocrit 0.17 L/L, reticulocyte count 1%. What are the absolute reticulocyte count and RPI? (Objective 1)
   A. absolute count, 25×10^{9}/L; RPI, 0.19
   B. absolute count, 250×10^{9}/L, RPI, 0.15
   C. absolute count, 170×10^{9}/L; RPI, 0.38
   D. absolute count, 100×10^{9}/L; RPI, 1.5
5. How would you classify the cell population with the following indices: MCV 70 fL, MCH 25 pg, MCHC 30 g/dL? (Objective 3)
   A. normocytic, normochromic
   B. macrocytic, normochromic
   C. microcytic, normochromic
   D. microcytic, hypochromic
6. Which of the following is a hemolytic anemia that would be classified as an extrinsic defect? (Objective 9)
   A. immune hemolytic anemia
   B. anemia caused by a membrane defect
   C. anemia associated with a deficiency of an erythrocyte enzyme
   D. anemia associated with a structurally abnormal hemoglobin that is unstable
7. Which of the following indicates that compensated hemolytic disease is present in a patient with increased erythrocyte destruction? (Objective 6)
   A. increased carboxyhemoglobin
   B. decreased hemoglobin
   C. increased hemoglobin
   D. normal hemoglobin
8. Which of the following is most typically found in a hemolytic anemia? (Objective 5)
   A. reticulocytopenia
   B. decreased IRF
   C. RPI >2
   D. increased M:E ratio in bone marrow
9. Upon examination of the stained blood smear from a patient with anemia and jaundice, the laboratory professional noted many spherocytes. This is an indication of: (Objective 9)
   A. an extrinsic defect
   B. extravascular hemolysis
   C. intravascular hemolysis
   D. inherited anemia
10. The clinical finding common in a patient with hemolytic anemia is (Objective 8)
    A. pica
    B. kidney stones
    C. jaundice
    D. lymph node enlargement

Level II

1. What follow-up test is most appropriate to determine the cause of anemia in a patient with the following results: RBC count 2.5×10^{12}/L, hemoglobin 5.3 g/dL, hematocrit 0.17 L/L, reticulocyte count 1%? (Objective 11)
   A. IRF
   B. absolute reticulocyte count
   C. serum ferritin
   D. RPI
2. What is the functional classification of the anemia in the preceding question if the serum ferritin is decreased? (Objective 8)
   A. proliferation defect
   B. survival defect
   C. nuclear maturation defect
   D. cytoplasmic maturation defect

3. Why might the serum bilirubin results be misleading as an indicator of erythrocyte destruction inpatients with microcytic hypochromic anemias? (Objective 2)

   A. The liver is not excreting the bilirubin due to liver failure.

   B. The cells are being produced at a lower rate than normal.

   C. Hypochromic cells do not release much hemoglobin; hence, less bilirubin is formed.

   D. The cells are not destroyed as fast in individuals with microcytic anemia so the bilirubin will be falsely decreased.

4. A patient has anemia, decreased haptoglobin, hemosiderinurea, and hemoglobinuria. The reticulocyte count is 10%. How would you classify this anemia? (Objectives 8, 9)

   A. hypoproliferative

   B. extravascular hemolytic

   C. maturation defect

   D. survival defect

5. In an untreated anemia caused by hemorrhage, what would you expect to find in the laboratory investigation? (Objective 2)

   A. presence of polychromatophilic macrocytes on the peripheral blood smear

   B. hypoplastic bone marrow with nuclear maturation abnormalities

   C. megaloblastosis in the bone marrow and pancytopenia in the peripheral blood

   D. decreased IRF and RPI

6. Bone marrow examination in a patient with a hemoglobin of 80 g/L reveals hypercellularity with a decreased M:E ratio and normal appearing erythrocytic precursors. This is an indication that the anemia is likely due to a: (Objective 5)

   A. proliferation defect

   B. nuclear maturation defect

   C. survival defect

   D. cytoplasmic maturation defect

7. Which of the following is an adaptation to anemia that tends to increase blood flow to tissues? (Objective 1)

   A. decrease in 2,3-DPG

   B. shallow inspiration

   C. decreased respiratory rate

   D. increased heart rate

8. Which of the following findings is characteristic of the bone marrow in a hemolytic anemia? (Objective 5)

   A. increased M:E ratio

   B. erythroid hyperplasia

   C. increased amount of fat

   D. hypoplasia

9. A 4-year-old boy has severe anemia. His x-rays reveal thinning cortical bone, and he had splenomegaly. This diagnosis and clinical symptoms indicate: (Objective 3)

   A. a chronic hemolytic process

   B. an acute hemolytic anemia

   C. intravascular hemolysis

   D. extravascular hemolysis

10. A patient has a hemoglobin of 90 g/L and a reticulocyte count of 20%. A bone marrow examination revealed a decreased M:E ratio and 70% cellularity. How would you describe this marrow? (Objective 4)

    A. aplasia

    B. erythrocytic hypoplasia

    C. dysplasia

    D. erythrocytic hyperplasia

11. A patient is suspected of having an autoimmune hemolytic anemia. Many spherocytes are present on the blood smear, and the reticulocyte count is 20%. What test should be performed to determine whether this is an autoimmune process? (Objective 10)

    A. serum bilirubin

    B. serum LD

    C. urinalysis

    D. AHG (DAT) test

12. What laboratory test result is suggestive of an intravascular hemolytic process? (Objectives 9, 11)

    A. decreased LDH

    B. decreased haptoglobin

    C. decreased reticulocyte count

    D. decreased bilirubin

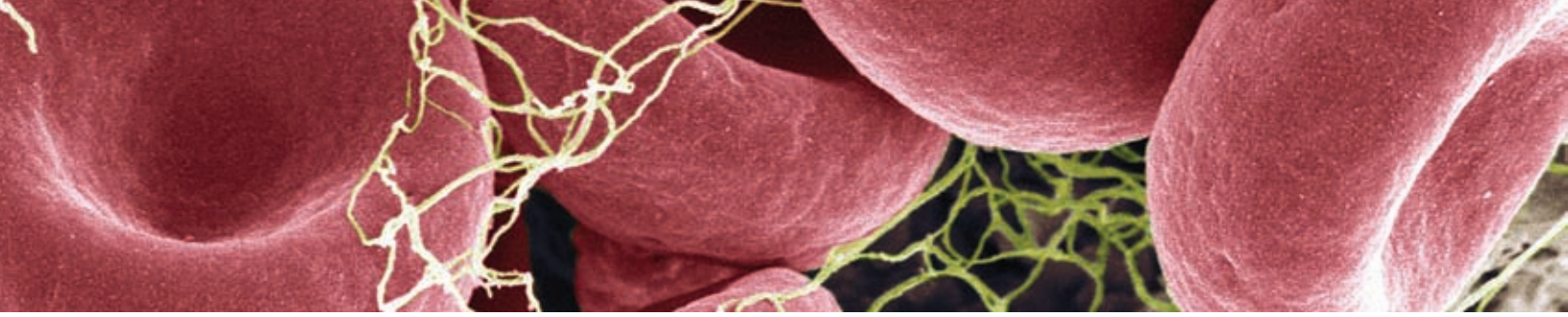
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Crosby WH. Reticulocyte counts. *Arch Intern Med*. 1981;141:1747–48.
2. Centers for Disease Control. CDC criteria for anemia in children and childbearing-aged women. *MMWR*. 1989;38:400–4.
3. Nordenberg D, Yip R, Binkin NJ. The effect of cigarette smoking on hemoglobin levels and anemia screening. *JAMA*. 1990;264:1556–59.
4. Hillman RS. Hershko, C. Acute blood loss anemia. In:, Lichtman MA, Beutler E, Kipps TJ et al., eds. *Hematology*, 7th ed. New York: McGraw-Hill; 2007: Chapter 54.
5. Kheir F, Haddad RY. Anemia in the elderly. *Disease-a-Month*. 2010;56:456–67.
6. Sheth TN, Choudhry NK, Bowes M et al. The relation of conjunctival pallor to the presence of anemia. *J Gen Intern Med*. 1997;12(2):102–6.
7. Stoltzfus RJ, Edward-Raj A, Dreyfuss ML et al. Clinical pallor is useful to detect severe anemia in populations where anemia is prevalent and severe. *J Nutrit*. 1999;129(9):1675–81.
8. Tabbara, IA. Hemolytic anemias: Diagnosis and management. *Med Clin N Amer*. 1992;76:649–68.
9. Goonewardene W, Shehata M, Hamad A. Anemia in pregnancy. *Best Prac Res Clin Obstet Gynaecol*. 2012;26:3–24.
10. Guralnik JM, Eisenstaedt RS, Ferrucci L et al. Prevalence of anemia in persons 65 years and older in the United States: evidence for a high rate of unexplained anemia. *Blood*. 2004;104:2263–68.
11. Steensma DP, Tefferi A. Anemia in the elderly: how should we define it, when does it matter, and what can be done. *Mayo Clin Proc*. 2007;82(2):958–66.
12. NCCLS. Methods for reticulocyte counting (flow cytometry and supravital dyes): Approved guideline. *NCCLS Document H44-A*. 1997;17(15).
13. Piva E, Brugnara C, Chlandetti L et al. Automated reticulocyte counting: state of the art and clinical applications in the evaluation of erythropoiesis. *Clin Chem Lab Med*. 2010;48(10):1369–380.
14. Kosower NS. Altered properties of erythrocytes in the aged. *Am J Hematol*. 1993;42:241–47.
15. Mark PW. Approach to anemia in the adult and child. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology Basic Principles and Practice*. 6th ed. Philadelphia: Elsevier, Churchill Livingstone; 2012:418–26.
16. Buttarello M, Bulian P, Farina G et al. Five fully automated methods for performing immature reticulocyte fraction. *Am J Clin Pathol*. 2002;117:871–79.
17. Chang CC, Kass L. Clinical significance of immature reticulocyte fraction determined by automated reticulocyte counting. *Am J Clin Pathol*. 1997;108(1):69–73.
18. Brugnara C, Schiller B, Moran J. Reticulocyte hemoglobin equivalent (Tet He) and assessment of iron-deficient states. *Clin Lab Haem*. 2006;28:303–8.
19. Djulbegovic B. Red blood cell problems. In: Djulbegovic B, ed. *Reasoning and Decision Making in Hematology*. Edinburgh, UK: Churchill Livingstone; 1992:13.
20. Masson C. Rheumatoid anemia. *Joint, Bone, Spine: Revue du Rhumatisme*. 2011;78:131–37. Review.
21. Beutler E, Luzzatto L. Hemolytic anemia. *Sem Hematol*. 1999;36(4 suppl 7):38–47.
22. Cunha BA, Nausheen S, Szalda D. Pulmonary complications of babesiosis: case report and literature review. *Cur J Clin Microbiol Infec Dis*. 2007;26:505–8.
23. Bessman JD, Gilmer PR, Gardner FH. Improved classification of anemias by MCV and RDW. *Am J Clin Pathol*. 1983;80:322–26.



12 Anemias of Disordered Iron Metabolism and Heme Synthesis

SHIRLYN B. McKENZIE, PhD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *sideropenic* as it relates to anemia.
2. Diagram the transport of iron from ingestion to incorporation into heme.
3. Define the following terms and explain their role in iron metabolism: *transferrin, hemosiderin, ferritin, total iron binding capacity (TIBC).*
4. Describe physiologic factors that affect the amount of iron needed by the body.
5. Compare and contrast the typical blood features and iron studies associated with iron-deficiency anemia (IDA), anemia of chronic disease (ACD), lead poisoning, and sideroblastic anemia.
6. Explain the etiology and pathophysiology of iron-deficiency anemia, anemia of chronic disease, and sideroblastic anemia.
7. Define *hemosiderosis.*
8. Calculate transferrin saturation and unsaturated iron-binding capacity (UIBC) given serum iron and TIBC.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. List the three stages of iron deficiency, and define characteristic RBC morphology of each stage.
2. Compare and contrast iron stores, hemoglobin, serum iron, TIBC, saturation, serum ferritin, and RBC morphology in the three stages of iron deficiency.
3. Describe the function of the proteins involved in iron metabolism including hepcidin, HFE, transferrin receptor, hemojuvelin, divalent metal transporter 1, duodenal cytochrome–B reductase, hephaestin, hypoxia-inducible factor, and ferroportin.
4. Explain the molecular control of total body iron and cellular iron.
5. Describe how genetic defects in the iron metabolism proteins can affect the iron homeostasis in the body.
6. Contrast the basic defects in iron-deficiency anemia, sideroblastic anemia, and anemia of chronic disease, and describe how each defect affects hemoglobin synthesis.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

7. Recognize the clinical features associated with iron deficiency.
8. Correlate the following laboratory features with iron-deficiency anemia, anemia of chronic disease, and sideroblastic anemia: erythrocyte morphology and protoporphyrin studies, iron studies, and bone marrow.
9. Select laboratory tests, and discuss test results that help differentiate iron-deficiency anemia, anemia of chronic disease, and sideroblastic anemia.
10. Summarize the results of bone marrow analysis in sideroblastic anemia and anemia of chronic disease, and contrast them with those found in iron-deficiency anemia.
11. Outline the classification of sideroblastic anemias, and describe the differentiating feature of the hereditary type.
12. Describe the relationship of the anemias associated with alcoholism and malignant disease to sideroblastic anemia.
13. Describe the role of molecular diagnostics in hereditary sideroblastic anemia.
14. Explain the significance of finding microcytic anemia in the presence of lead poisoning, and suggest reflex testing that would help determine an accurate diagnosis.
15. Explain how lead poisoning and alcohol affect erythropoiesis and their relationship to sideroblastic anemia, and recognize the abnormal peripheral blood and clinical features that can be associated with these disorders.
16. Discuss the treatment for iron-deficiency anemia, sideroblastic anemia, and anemia of chronic disease and expected laboratory findings associated with successful therapy.
17. Differentiate primary (hereditary) and secondary hemochromatosis and summarize typical results of iron studies in these conditions.
18. Describe the genetic abnormalities and pathophysiology of hereditary hemochromatosis, and identify the screening and diagnostic tests for this disease.
19. Describe the basic defect in porphyria and its effect on the blood.
20. Develop a reflex-testing pathway for an efficient and cost-effective diagnosis when microcytic and/or hypochromic cells are present.
21. Evaluate laboratory test results, and use them to identify the etiology and pathophysiology of the anemias that have a defective heme synthesis component.

Key Terms

Anemia of chronic disease (ACD)
Anemia of inflammation (AI)
Apoferritin
Apotransferrin
Ceruloplasmin
DcytB
DMT1
Ferritin
Ferroportin 1
Hemochromatosis
Hemojuvelin (HJV)
Hemosiderin
Hepcidin
Hephaestin
HFE
Hypoxia-inducible factor (HIF)
Iron-deficiency anemia (IDA)
Pappenheimer body
Pica
Plumbism
Porphyria
Serum transferrin receptor (sTfR)
Sideroblastic anemia
Sideropenic anemia
Total iron-binding capacity (TIBC)
Transferrin (Tf)
Transferrin receptor1 (TfR1)
Transferrin receptor2 (TfR2)
Transferrin saturation
Unsaturated iron-binding capacity (UIBC)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Diagnosis of anemia: List the laboratory tests used to diagnose and classify anemias, and identify abnormal test values. (Chapters 10, 11)
- Classification of anemia: Outline the morphologic and functional classification of anemias. (Chapter 11)

Level II

- Function, structure, and synthesis of hemoglobin: Diagram the synthesis of heme and explain the role of iron in hemoglobin synthesis. (Chapter 6)
- Erythrocyte destruction: Diagram degradation of hemoglobin when the erythrocyte is destroyed and interpret laboratory tests associated with increased erythrocyte destruction. (Chapters 5,11)

CASE STUDY

We will address this case study throughout the chapter.

Jose, an 83-year-old anemic male, was admitted to a local hospital with recurrent urinary tract bleeding and an infection associated with prostatitis.

1. How can these conditions affect the hematopoietic system?

OVERVIEW

This chapter includes a discussion of a group of anemias associated with defective hemoglobin synthesis due to faulty iron metabolism or porphyrin biosynthesis. The discussion begins with a detailed description of iron metabolism and laboratory tests used to assess the body's iron concentration. This is followed by a description of the specific anemias included in this group—iron-deficiency anemia, anemia of chronic disease, and sideroblastic anemia. Hemochromatosis is also discussed even though it is usually not characterized by anemia. In hemochromatosis, iron metabolism is abnormal, and results of iron

studies must be differentiated from those found in sideroblastic anemia. The rare porphyrias are discussed briefly because porphyrin is an integral component in the synthesis of heme.

INTRODUCTION

Defective hemoglobin production can be due to disturbances in either heme or globin synthesis (Table 12-1 ★). The result of these disturbances is an erythrocyte cytoplasmic maturation defect often reflected by a microcytic, hypochromic anemia. Defective heme synthesis is caused by abnormalities of iron homeostasis (deficiency and/or metabolism) or rarely by defective porphyrin metabolism (Figure 12-1 ■). Defective globin synthesis is a result of deletions or mutations of globin genes. Globin gene deletions that result in decreased synthesis of globin are known as *thalassemia* (Chapter 14). Mutations that result in structurally abnormal globin chains are known as hemoglobinopathies, which are not usually associated with microcytic hypochromic erythrocytes (Chapter 13).

Anemia characterized by deficient iron for hemoglobin synthesis is known as **sideropenic anemia**, more commonly referred to as **iron-deficiency anemia (IDA)**. Iron deficiency primarily affects the erythrocyte and developing central nervous system. Sideropenic anemia caused by inadequate iron intake or absorption or increased blood loss responds to iron therapy given either orally, or less commonly, parenterally.

Anemia also can result from defective iron metabolism. Adequate or excess stores of iron but defective iron utilization in the synthesis of hemoglobin characterize these anemias (functional iron deficiency). The **anemia of chronic disease (ACD)**, also referred to as the **anemia of inflammation (AI)**, is included in this group, and is characterized by iron retention in the macrophages, thus making iron unavailable to the erythrocyte for heme synthesis.

Defects in porphyrin synthesis involve the enzymes required for heme synthesis. The defect can affect the insertion of iron into the porphyrin ring to form heme. These conditions include primary and secondary **sideroblastic anemias**. The **porphyrias** are included in this chapter, although except for erythropoietic porphyria, they are not generally characterized by the presence of anemia.

★ TABLE 12-1

Causes of Defective Hemoglobin Production That Could Result in a Microcytic Hypochromic Anemia

Defects in heme synthesis
- Abnormal iron metabolism
  - Iron deficiency
  - Defective iron utilization
- Defective porphyrin metabolism

Defects in globin synthesis (thalassemias): deletions or mutations of globin genes

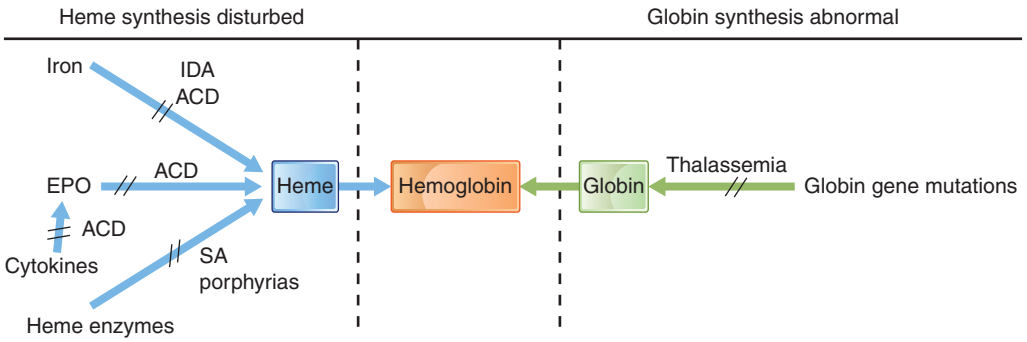
IRON METABOLISM

Every cell in the body requires iron, which has vital roles in oxidative metabolism, cellular growth and proliferation, and oxygen transport and storage.[1] Iron must be bound to protein compounds to fulfill these functions. Iron in inorganic compounds or in an ionized form is potentially dangerous. If the amount of iron exceeds the body's capacity for transport and storage in the protein-bound form, iron toxicity can develop, causing damage to cells and a potentially lethal condition. Conversely, if too little iron is available, the synthesis of physiologically active iron compounds is limited, and critical metabolic processes are inhibited.

Iron cannot freely diffuse across membranes but requires special transport involving a variety of proteins. Enterocytes (absorptive cells at the luminal [apical] surface of the duodenum), hepatocytes, and macrophages can import and export iron. On the other hand, erythrocyte precursors use most, if not all, of the imported iron and do not export it.[1] Important advances in our understanding of iron metabolism are the result of the discovery of genes and proteins that participate in regulating iron homeostasis. As the roles of the proteins are discovered, the pathophysiology of disorders involving iron metabolism is revealed.

Distribution

Iron-containing compounds in the body are one of two types: (1) functional compounds that serve in metabolic functions (hemoglobin, myoglobin, iron-responsive element-binding protein) or



■ **FIGURE 12-1** Sites of defective hemoglobin synthesis that can result in anemia. Some of these anemias are hereditary or congenital and others are acquired.

ACD = anemia of chronic disease; IDA = iron-deficiency anemia; SA = sideroblastic anemia; EPO = erythropoietin; // = defective or disturbed

★ **TABLE 12-2** Composition and Distribution of Iron in Adults

Compound	Iron Content, Male (mg Iron/Kg Body Weight)	Iron Content, Female (mg Iron/Kg Body Weight)	Percent of Total Iron
Functional Iron			
Hemoglobin	31	28	60–75.0
Myoglobin	5	4	3.5
Other tissue iron	<1	<1	0.2
Heme enzymes (cytochromes, catalases, peroxidases)			
Nonheme enzymes (iron-sulfur proteins, metalloflavoproteins, ribonucleotide reductase)			
Transport			
Transferrin	<1	<1	0.1
Storage			
Ferritin	~8	~4	10–20
Hemosiderin	~4	~2	5–10
Labile pool	~1	~1	2
Total iron	~50	~40	

enzymatic functions (cytochromes, cytochrome oxygenase, catalase, peroxidase) and (2) compounds that serve as transport proteins (transferrin, transferrin receptor) or storage depots for iron (ferritin and hemosiderin) (Table 12-2 ★). A poorly understood iron compartment is the intracellular "labile pool." Iron leaves the plasma and enters the intracellular fluid compartment for a brief time before it is incorporated into cellular components (heme or enzymes) or storage compounds. This labile pool is believed to be the chelatable iron pool (see the section on "Therapy for Hemochromatosis"). The total iron concentration in the body is 40–50 mg of iron/kg of body weight. Men usually have higher amounts than women.

Iron is found primarily in erythrocytes, macrophages, hepatocytes, and enterocytes. Hemoglobin constitutes the major fraction of body iron (functional iron) with a concentration of about 1 mg iron/mL erythrocytes. Iron in hemoglobin remains in the erythrocyte until the cell is removed from the circulation. Hemoglobin released from the erythrocyte is then degraded in the macrophages of the spleen and liver, releasing iron. Approximately 85% of this iron from degraded hemoglobin is promptly recycled from the macrophage to the plasma where it is bound to the transport protein, transferrin, and delivered to developing erythroblasts in the bone marrow for heme synthesis. The macrophages recycle 10–20 times more iron than is absorbed in the gut.[2] Thus, iron recycling provides most of the marrow's daily iron requirement for erythropoiesis (Chapter 5).

Iron in hepatocytes and intestinal enterocytes is stored and utilized as needed to maintain iron homeostasis. The hepatocytes store iron that can be released and utilized when the amount of iron in the plasma is not sufficient to support erythropoiesis. Enterocytes that absorb dietary iron can either export it to the plasma or store it.

Absorption

Total body iron homeostasis depends on balancing and linking the absorption of iron by the enterocytes of the duodenum with total body requirements. No significant mechanism exists to effect iron loss. Factors influencing iron absorption are listed in Table 12-3 ★.

★ **TABLE 12-3** Factors Affecting Iron Absorption in the GI Tract

- Bioavailability of iron: from the diet and macrophage recycling
- Condition of mucosal cells in the GI tract
- Intraluminal factors: parasites, toxins, intestinal motility; increased motility or decreased absorptive surface area can decrease absorption
- Hematopoietic activity of bone marrow: rate of erythropoietic activity is directly related to the amount absorbed
- Tissue iron stores: amount of iron absorbed is inversely related to the amount of storage iron
- Oxygen content of the blood: hypoxia is associated with increased absorption
- Systemic inflammation or infection: decreases absorption
- Blood hemoglobin concentration: anemia is associated with increased absorption

Dietary iron exists in two forms: nonheme iron (ionic or ferric form, Fe^{+++}) present in vegetables and whole grains and heme iron (ferrous form, Fe^{++}) present primarily in red meats in the form of hemoglobin. Nonheme iron is the most common form ingested worldwide; heme iron is more common in Western countries (providing about 10–15% of daily iron requirements). The ferric complexes from nonheme sources are not easily absorbed. Gastric acid solubilizes this form of iron and provides an acidic environment around the apical brush border of the enterocytes. This low pH facilitates the transport of iron across the enterocyte membrane by membrane transporters. The ferric iron is reduced to the ferrous state at the enterocyte brush border by the enzyme, Duodenal cytochrome b **(DcytB)**, a ferric reductase. The ferrous iron is then transported across the enterocyte apical plasma membrane by divalent metal transporter1 **(DMT1)**, an integral membrane protein. DMT1 also transports the divalent forms of manganese, lead, zinc, cobalt, and copper across the enterocyte membrane[3] (Figure 12-2 ■). This membrane iron transport depends on the inward transport of protons.[1]

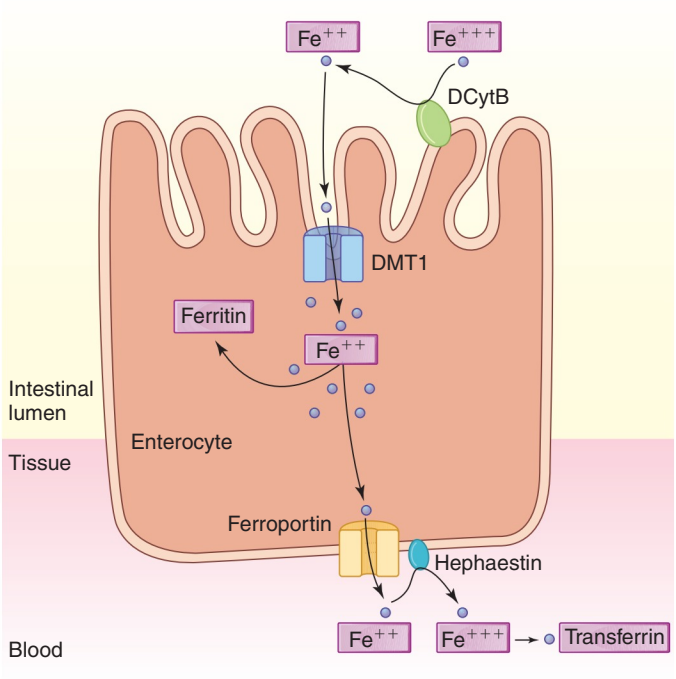


FIGURE 12-2 The absorption of nonheme iron in the intestine. Most nonheme iron in the diet is in the ferric iron form (Fe^{+++}). When the Fe^{+++} reaches the intestine and comes into contact with the cells lining the gut (enterocytes), the iron is reduced to the ferrous form (Fe^{++}) by a reductase, DCytB, located at the apical enterocyte membrane. The Fe^{++} then can be transported across the membrane by DMT1. Ferroportin transports the iron across the enterocyte basolateral membrane, a process thought to be facilitated by hephaestin. Hephaestin is an oxidase that oxidizes the iron to Fe^{+++}, the form that combines with transferrin. Some iron can remain in the cell as ferritin, depending on the systemic iron balance.

Heme iron is more readily absorbed than nonheme iron, but the mechanism of absorption is less well understood. Heme is split from the globin portion of hemoglobin in the intestine and is then assimilated directly by the enterocytes. Once inside the cell, iron is released from heme by heme oxygenase. The iron then enters the same iron pool as the nonheme iron. A heme carrier protein (HCP1) found in the duodenal enterocyte was recently described, but its contribution to iron absorption has not been documented. HCP1 appears to transport folate more efficiently than heme.[4,5]

In the enterocyte, the iron can be stored as ferritin or transported across the basolateral membrane into the plasma. The iron stored as ferritin is lost when the enterocyte is sloughed off into the intestinal tract. Iron transport across the basolateral membrane is via the basolateral transporter protein **ferroportin 1** (also known as *IREG1*). Ferroportin 1, an integral membrane protein, transports ferrous ions and is the only known cellular exporter of iron. Export is facilitated by the ferroxidase, **hephaestin** (a homologue of the plasma protein **ceruloplasmin**). Hephaestin oxidizes the Fe^{++} to Fe^{+++}, the form of iron required for binding to apotransferrin in the blood. Hephaestin is a copper-containing ferroxidase that requires adequate amounts of copper for its function. Thus, it is not surprising that copper deficiency is associated with abnormal iron metabolism. Export of iron from nonintestinal cells, including macrophages, requires ceruloplasmin, which also converts Fe^{++} to Fe^{+++} for binding to transferrin.

There appears to be a predetermined set point of iron stores that results in a negative correlation between the amount of iron absorbed and the amount of iron stored.[6] The efficiency of intestinal absorption of iron increases in response to accelerated erythropoietic activity and depletion of body iron stores. Bleeding, hypoxia, or hemolysis results in accelerated erythrocyte production and enhanced absorption of iron. However, increased iron uptake in extravascular hemolytic anemias and anemias associated with a high degree of ineffective erythropoiesis can lead to an excess accumulation of iron in various organs because the body does not lose the iron from erythrocytes hemolyzed in vivo. Conversely, diminished erythropoiesis as occurs in starvation decreases the absorption of iron.

Iron-deficiency anemia (IDA) from a lack of dietary iron is usually treated with daily oral doses of ferrous salts. The efficiency of absorption of this therapeutic iron is greatest during the initial treatment period when body stores are depleted. Increased absorption occurs up to 6 months after hemoglobin values return to normal or until iron stores are replenished. Absorption also increases 10–20% in early stages of developing iron deficiency (ID).

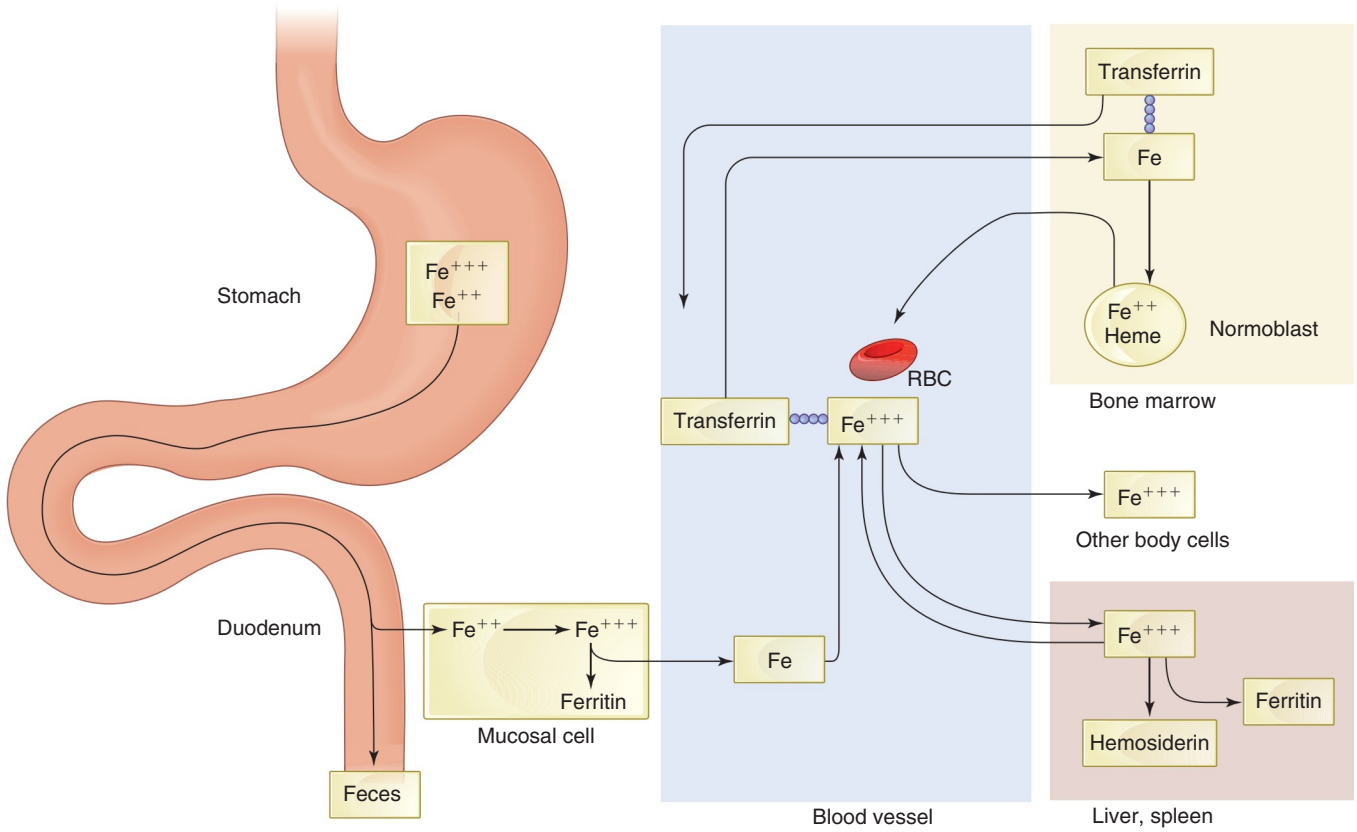
Transport

Transferrin (Tf) is a plasma iron transport protein that mediates iron exchange between tissues (Figure 12-3 ■). It is not lost in delivering iron to the cells but returns to the plasma and is reused. Transferrin is a single polypeptide chain composed of two homologous lobes, each of which contains a single iron-binding site. The binding of a ferric iron to either binding site is random. If only one transferrin lobe binds an iron molecule, it is termed *monoferric transferrin*; if both sites are occupied, it is *diferric transferrin* (also called holo-transferrin). Transferrin without iron is called **apotransferrin**.

Each gram of transferrin binds 1.4 mg of iron. Enough transferrin is generally present in plasma to bind 250–450 mcg (μg) of iron per deciliter of plasma. This is referred to as the **total iron-binding capacity (TIBC)**.

The concentration of iron in the blood is primarily determined by the amount of iron released from macrophages to plasma transferrin and the amount extracted from the plasma by developing erythroid precursor cells. The serum iron concentration is about 50–180 mcg/dL and almost all (95%) of this iron is complexed with transferrin; thus, transferrin is about one-third saturated with iron (serum iron/TIBC × 100 = % **transferrin saturation**). Clinically, the transferrin saturation is a good indicator of the amount of iron available for erythropoiesis. The reserve iron-binding capacity of transferrin (transferrin without bound iron) is referred to as the serum **unsaturated iron-binding capacity (UIBC)** (TIBC − serum iron = UIBC). The majority of transferrin-bound iron is delivered to the developing bone marrow erythroblasts for use in hemoglobin synthesis. Iron in excess of physiologic requirements is deposited in tissues (primarily the liver) for storage.

Only a small amount of transferrin-bound iron is derived from iron absorbed by enterocytes. Most of the iron bound to transferrin is recycled from the monocyte-macrophage system. The major flow of iron in the body is from macrophages to erythroid marrow, to



■ **FIGURE 12-3** Iron is absorbed by the mucosal cells in the gut as ferrous iron. It can be stored in the mucosal cell as ferritin, or it can leave the cell as oxidized ferric iron and be transported in the blood by transferrin. Transferrin can deliver iron to developing erythroblasts in the bone marrow, other body cells, or macrophages in the liver or spleen. Transferrin is reutilized after it delivers iron to the cells.

erythrocytes, and back, to macrophages when the senescent erythrocyte is removed and degraded by liver, bone marrow, and splenic macrophages. Recovered iron from hemoglobin catabolism in the monocyte-macrophage system enters the plasma and is again bound to transferrin for transfer back to the bone marrow (Figure 12-4 ■). In disorders associated with intravascular hemolysis in which hemoglobin is released into the plasma, plasma hemoglobin combines with haptoglobin (Chapter 6). The haptoglobin–hemoglobin complex is taken into the macrophage via the cell's hemoglobin scavenger receptor, CD163.[7]

In contrast to serum ferritin, transferrin is a negative acute phase reactant (levels decrease during the acute phase response). Increased levels are found during pregnancy and in estrogen therapy use.

Lactoferrin also functions as an iron transport protein but is found primarily in tissue fluid and cells. It has antimicrobial properties and is important in protecting the body from infection.

Transferrin releases iron at specific receptor sites on cells, referred to as **transferrin receptor1 (TfR1)**. These receptors are expressed on virtually all cells, but the number per cell is a function of cellular iron requirements. Cells with high iron requirements have high numbers of TfR1. Erythroid precursors, especially intermediate erythroblasts that are rapidly synthesizing hemoglobin, have high numbers of transferrin receptors, about 800,000 per cell. The TfR1 is a transmembrane glycoprotein dimer with two identical subunits, each of which can bind a molecule of transferrin. A homologous protein, **transferrin receptor 2 (TfR2)**, is more limited in expression and can also bind transferrin but with less affinity than TfR1. TfR2 is found predominantly on hepatocytes, duodenal crypt cells, as well as erythroid cells. TfR2 interacts with HFE to regulate hepcidin synthesis and thus has a role in regulating total body iron homeostasis (see subsequent discussion, "Systemic Iron Balance, HFE").

Iron enters the cell in an energy- and temperature-dependent process. After transferrin binds to its receptor, the transferrin–TfR complex clusters with other transferrin–TfR complexes on the cell membrane, and the membrane invaginates and reseals, forming an endosome with the complex inside (endocytosis) (Figure 12-5 ■). In the acidic endosome, iron is released from transferrin and transported into the cytoplasm via DMT1 transport protein present in the endosomal membrane. The endosome with the apotransferrin and TfR is transported back to the cell surface. The apotransferrin is released, making both it and the receptor available for recycling. When the concentration of plasma iron exceeds the transferrin binding capacity, the hepatocytes can take up the unbound iron directly. The mechanism of this uptake is unknown but appears to be distinct from that involving DMT1.

Cells release the extracellular portion of their transferrin receptors through proteolytic cleavage as they mature. These cleaved receptors, referred to as **serum or soluble transferrin receptors (sTfR)**, are found in the blood in proportion to the erythrocyte mass. With increased erythropoiesis, including ineffective erythropoiesis, the concentration of sTfR in the plasma increases.

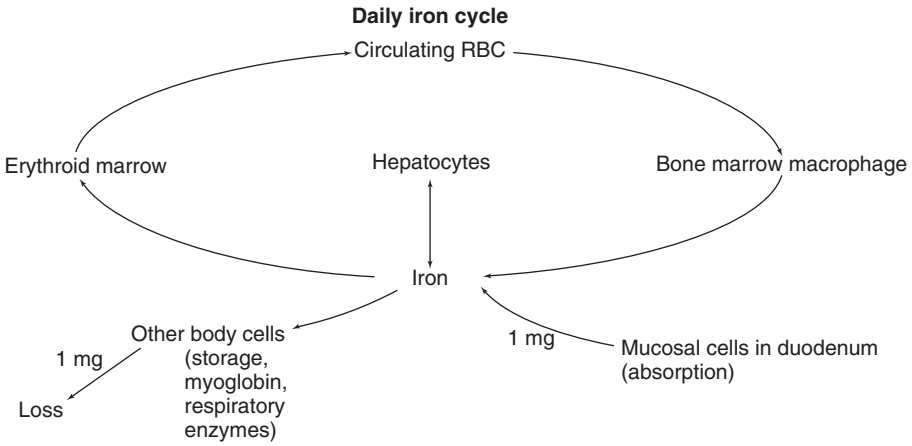


FIGURE 12-4 The daily iron cycle. Most iron is recycled from the erythrocytes to macrophages in the bone marrow. Macrophages release iron as needed to the developing erythroid cells in the bone marrow. Only a small amount of iron is lost from the body through loss of iron-containing cells. To maintain iron balance, a similar amount of iron is absorbed from the duodenum.

Storage

The primary iron storage depot is the liver. The largest nonheme iron stores in the body are hemosiderin and ferritin (Table 12-4 ★). Storage iron provides a readily available iron supply in the event of increased iron loss through bleeding. Depletion of these storage compounds reflects an excess iron loss over what is absorbed.

Ferritin

Ferritin consists of a spherical protein shell that can store up to 4500 molecules of Fe^{+++} iron. Ferritin is 17–33% iron by weight; without iron, it is called **apoferritin**.

Ferritin is a multimer composed of 24 subunits arranged to form a hollow sphere. There are two types of subunit polypeptides, heavy (H) and light (L). The H polypeptide has ferroxidase activity, but the L form does not. The proportion of H and L subunits in ferritin varies by cell type. Ferritins in the heart, placenta, and erythrocytes are rich in the H subunit, and ferritins in the iron storage sites, such as the liver and spleen, are rich in L subunits.

Ferritin acts as the primary storage compound for the body's iron and is readily available for erythropoiesis. It controls the amount of iron released for cellular activity and, by binding the iron, protects the cellular constituents from oxidative damage catalyzed by free ferrous ions. Ferritin is found in the bone marrow, liver, and spleen, usually within membrane-bound vesicles called *siderosomes*. Mature erythrocytes usually do not contain iron particles because any excess iron in the cell after hemoglobin synthesis is complete is removed by splenic macrophages.

Ferritin is a water-soluble form of storage iron that cannot be visualized by light microscopy on unstained specimens but can stain with iron stains (when clustered in siderosomes). Ferritin is primarily an intracellular protein, but small amounts enter the blood through active secretion or cell lysis (serum ferritin). Secreted ferritin differs from intracellular ferritin in that it is glycosylated and relatively iron poor. The amount of serum ferritin parallels the concentration of storage iron in the body. Therefore, serum ferritin concentration is used as an index of iron stores: 1 ng/mL of serum ferritin indicates about 8 mg of storage iron. Serum ferritin does not exhibit diurnal variations as are seen with serum iron levels.

The patient's comorbidities must be considered when interpreting serum ferritin levels. Ferritin is not a reliable indicator of iron stores in the presence of inflammation or tissue damage because it is an acute phase reactant and thus increases in these conditions. However, if serum ferritin is decreased, it invariably means that iron stores are low or depleted.

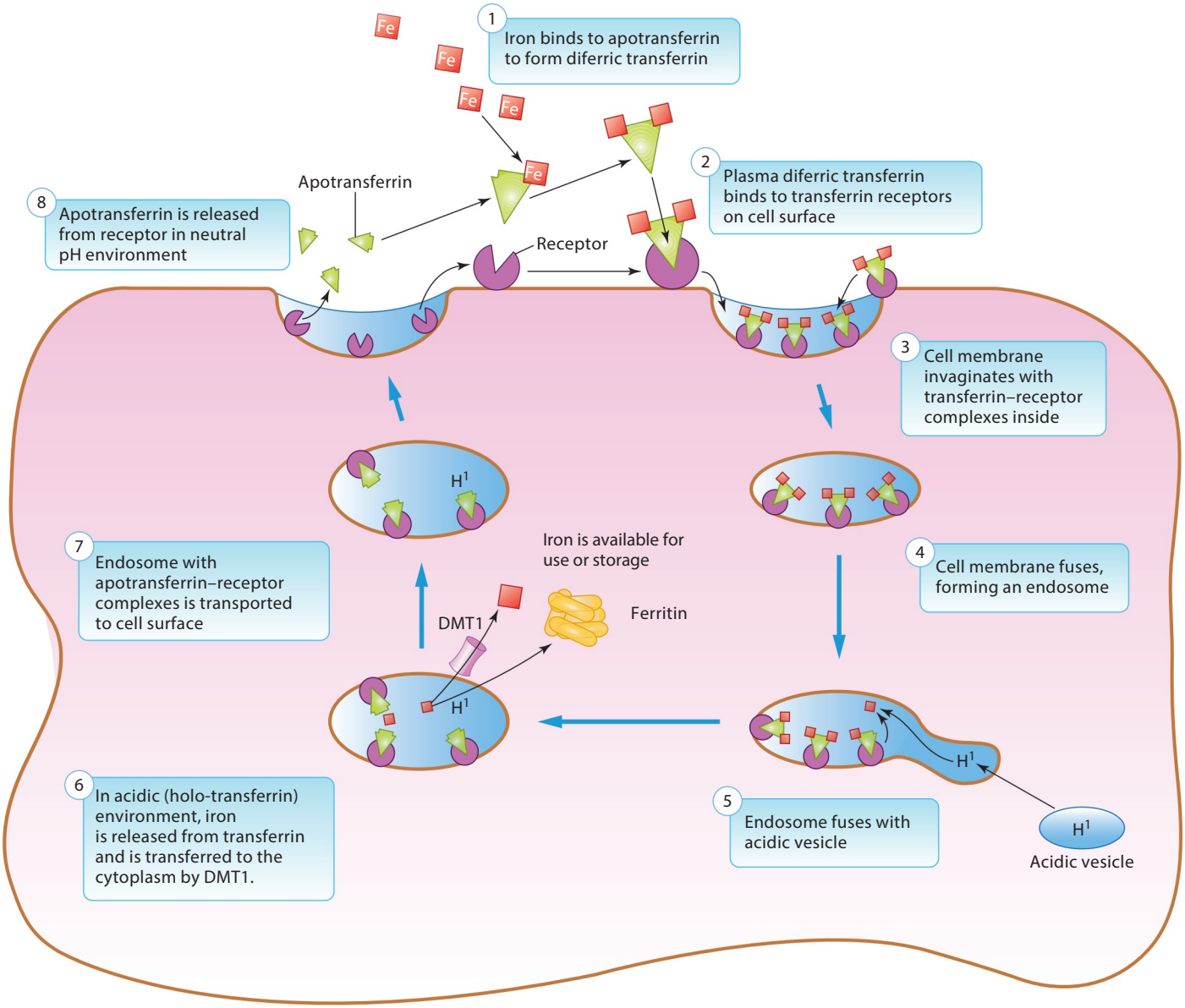
Hemosiderin

Hemosiderin is a heterogeneous aggregate of carbohydrate, lipid, protein, and iron; up to 50% of its weight is iron. Hemosiderin is found primarily in macrophages and is formed by the partial degradation of ferritin. At high levels of cellular iron, ferritin forms aggregates that are taken up by lysosomes and degraded, forming hemosiderin. The ratio of ferritin to hemosiderin varies with the total body iron concentration. At lower cellular iron concentrations, ferritin predominates, but at higher concentrations, the majority of storage iron exists as hemosiderin. Iron from hemosiderin is released slowly and is not readily available for cellular metabolism. Iron binding in the form of hemosiderin also probably keeps iron from harming cellular constituents.

Hemosiderin can be estimated on bone marrow tissue sections. Bone marrow macrophages contain hemosiderin if body iron stores are normal or increased. Hemosiderin appears as yellow to brown refractile pigment on unstained marrow or liver specimens. On Prussian blue-stained specimens, the iron appears as blue intracellular particles. Stores can be graded from 0 to 4+ or as markedly reduced, normal, or increased (Chapter 38). Normally, from 20–60% of the erythroblasts contain stainable iron deposits.

Physiological Regulation of Iron Balance

Body iron is stringently conserved by recycling so that daily absorption and loss are small. Total body iron lost through secretions of urine, sweat, bile, and desquamation of cells lining the gastrointestinal tract amounts to about 1 mg/day. Normal erythrocyte aging results in destruction of 20–25 mL of erythrocytes/day (releasing about 20–25 mg iron), but most of this hemoglobin iron is scavenged and reused by developing erythroblasts. Thus, the total daily requirement for new iron is about 1 mg (Chapter 5).



■ **FIGURE 12-5** Cellular iron supply and storage. (1) Iron binds to apotransferrin in the plasma forming monoferric or diferric transferrin. (2) Transferrin binds to transferrin receptors on the cell surface (3). The transferrin–receptor complex enters the cell and the cell membrane fuses forming an endosome (4). An acidic vesicle fuses with the endosome (5) which results in the release of iron (6). The apotransferrin–receptor complex is transported to the cell surface (7) where the apotransferrin is released from the receptor and enters the plasma for reutilization (8).

Because there is no physiological route for excretion of excess iron, the major regulation of total body iron depends on accurate sensing of systemic iron and adjusting iron absorption and retention according to needs. Iron deficiency can occur if dietary intake of iron is not adequate, if absorption is impaired, or if there is increased loss of iron through bleeding. Iron overload can occur if absorption abnormally increases or if the individual receives transfusions or iron injections (Table 12-5 ★).

★ **TABLE 12-4** Storage Forms of Iron

Iron Form	Role	Laboratory Analysis	Reference Interval
Ferritin	Primary storage form of soluble iron; readily released for heme synthesis	Serum ferritin levels	20–300 mcg/L males 12–200 mcg/L females
Hemosiderin	Partially degraded storage form of iron; slow release	Bone marrow estimation using Prussian blue stain	20–60% sideroblasts in bone marrow

★ **TABLE 12-5** Causes of Iron Deficiency and Iron Overload

Causes of Iron Deficiency	Causes of Iron Overload
• Increased requirement/demand due to blood loss, rapid growth, treatment with EPO • Inadequate diet • Malabsorption	• Increased absorption of iron • Multiple transfusions • Iron injections

Systemic Iron Balance

Iron homeostasis is accomplished by the interaction of iron with proteins that aide in its absorption, retention, export, and transport. These proteins include hepcidin, DcytB, DMT1, ferroportin, HFE, TfR1, TfR2, GDF15, HIF2, BMP, ceruloplasmin, and hemojuvelin (HJV) (Table 12-6 ★). The liver is the major site of expression of most of these iron-regulatory proteins. It also is the primary storage depot for iron and plays a central role in the regulation of total body iron homeostasis by synthesizing hepcidin in response to multiple signals.[2] In turn, hepcidin acts on hepatocytes, macrophages, and enterocytes to regulate how much iron they release into the plasma. Thus, the liver has been called the *command central* of iron homeostasis. The proteins involved in iron absorption and release can be upregulated or downregulated depending on total body iron status.

- **Hepcidin**, synthesized in the liver, is the master iron-regulating hormone whose expression is inversely related to total body iron demand. It regulates how much iron is absorbed and released into the plasma from enterocytes, hepatocytes, and macrophages. Hepcidin decreases iron absorption into the body by binding to and inducing degradation of ferroportin (the main cellular iron exporter in mammals), thus blocking basolateral iron export from the enterocyte. The iron is lost when these enterocytes are shed into the lumen of the intestine. Hepcidin also blocks the export of iron from the macrophages and hepatocytes resulting in retention of iron in these storage sites and a decrease in serum iron when hepcidin levels are increased. Because ferroportin determines whether iron is delivered to the plasma or remains within the enterocyte, hepatocyte, or macrophage, it plays a vital role in iron homeostasis. Hepcidin's interaction with ferroportin is thought to be the major mechanism that controls systemic iron homeostasis.[8]

Hepcidin expression is modulated by four conditions: iron stores, erythropoiesis, hypoxia, and inflammation/infection.[9] Hepcidin synthesis is induced by an excess of iron and is inhibited by lack of iron. The body's response to insufficient tissue oxygen delivery is an increase in erythropoiesis. An increase in erythropoietic activity results in a decrease in hepcidin synthesis, allowing the absorption of more iron in the intestine and increased release of iron from enterocytes, macrophages, and hepatocytes. The repressive effect of erythropoiesis on hepcidin synthesis is especially noted when erythroid precursors massively expand but undergo apoptosis before maturing to erythrocytes (ineffective erythropoiesis). It has been shown that these erythroid precursors release growth differentiation factor-15 (GDF-15) during cellular stress or apoptosis, which may be linked to low hepcidin expression and iron loading in conditions that have ineffective erythropoiesis.[10]

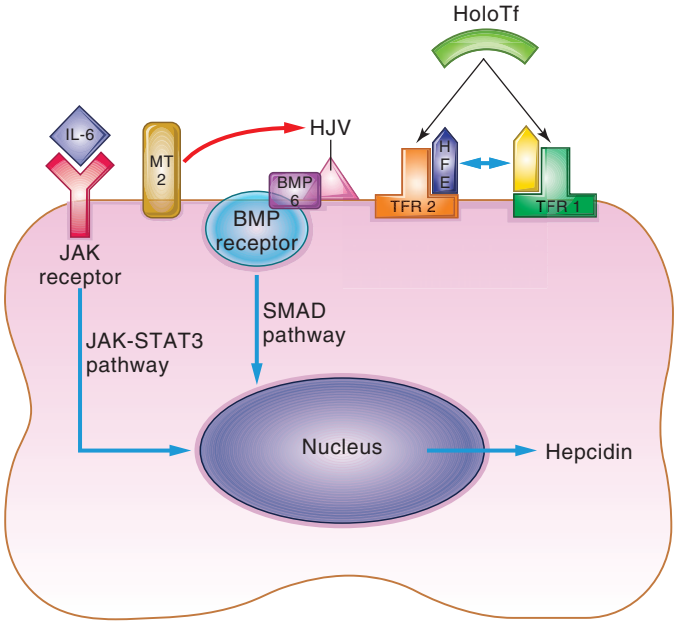
Hypoxia stimulates erythropoietin (EPO) production, resulting in increased erythropoiesis, which increases the demand for iron. Hypoxia-inducible factor-2 (HIF-2) is the transcription factor that regulates EPO synthesis as well as a spectrum of other hypoxic responses. HIF-2 signaling is activated under hypoxic conditions. HIF-2 binds to specific DNA consensus sequences referred to as hyypoxia-response elements (HFEs) and induces transcription of oxygen-sensitive genes. In addition to regulating EPO synthesis, HIF-2 plays a role in iron metabolism by directly regulating (increasing the synthesis of) DMT1 and DcytB, resulting in increased iron

★ **TABLE 12-6** Proteins Involved in Iron Homeostasis

Protein	Metabolic Role
Hepcidin	Master iron-regulating protein; regulates iron recycling/balance via interaction with ferroportin1; negative regulator of intestinal iron absorption; additional proteins influence synthesis
DMT1	Transports iron across the enterocyte apical plasma membrane; transports iron across endosome into cytoplasm of cells
DCytB	Reduces Fe^{+++} iron to Fe^{++} at enterocyte apical border
Ferroportin1	Exports cellular iron from enterocytes, macrophages, hepatocytes
Hephaestin	Facilitates cellular export of iron by ferroportin; oxidizes Fe^{++} iron to Fe^{+++} for binding to apotransferrin
Transferrin receptor1 (TfR1)	Binds diferric-transferrin to the cell for internalization of iron; can form molecular complex with HFE
Transferrin receptor2 (TfR2)	Forms molecular complex with HFE to regulate hepatic hepcidin expression; can bind transferrin
HFE	Interacts with TfR1 and TfR2 to control hepcidin expression
Transferrin (Tf)	Transports iron found in blood; diferric transferrin binds to TfR1 and can displace HFE from TfR1 when level of diferric transferrin increases
Hemojuvelin (HJV)	Regulates hepcidin expression
Ceruloplasmin	Copper containing ferroxidase; plays same role in iron export from macrophages and other nongut tissues as hephaestin does in enterocytes
GDF-15	Member of the transforming growth factor-β superfamily released from erythroblasts and other cells under stress and apoptosis; suppresses hepcidin synthesis; increased during ineffective erythropoiesis
HIF-2	Transcription factor involved in oxygen-dependent gene regulation; regulates EPO production and expression of DMT, DcytB, transferrin, TfR1, ceruloplasmin, and heme-oxygenase-1

absorption in the gut.[11] Other genes regulated by HIF-2 are transferrin, TfR1, ceruloplasmin, and heme-oxygenase-1. It may also play a role in hepcidin production.

Hepcidin synthesis is affected by not only iron status, hypoxia, and erythropoietic activity but also infection and inflammation. Bacteria-activated macrophages and neutrophils synthesize hepcidin but at lower levels than the hepatocytes. In the presence of inflammation, the cytokine interleukin-6 (IL-6) induces synthesis of hepcidin (through transcriptional regulation of the JAK-STAT3 signaling pathway) (see Chapter 4, Signaling Pathways and Transcription Factors). The increase in hepcidin results in hypoferremia (decreased serum iron) (Figure 12-6 ■).[12] Because hepcidin induces iron retention in the macrophages (traps intracellular iron) and most iron used in erythropoiesis comes from iron recycled from the macrophage, hypoferremia can develop rapidly in the presence of inflammation or infection even though total body iron can be normal or even increased. This appears to be the pathologic basis for the anemia of chronic disease (ACD) (see "Anemia of Chronic Disease").



■ **FIGURE 12-6** A model for molecular regulation of hepcidin. Hepcidin is regulated by at least two pathways: the iron-sensing pathway and the inflammatory pathway. The iron-sensing pathway involves the interaction of HFE with TfR1 and TfR2. Both receptors can bind HFE and diferric transferrin (holoTf). When the concentration of holoTf increases, it displaces HFE from TFR1 and both HFE and holoTf bind to TfR2. The HFE/TfR2/holoTf complex then interacts with a second protein complex (the bone morphogenetic protein [BMP] receptor, its ligand BMP6, and HJV coreceptor) to signal upregulation of hepcidin through the cell signaling SMAD pathway. Matriptase-2 (MT-2), a transmembrane serine protease, degrades HJV, impairing BMP receptor activation and decreasing hepcidin synthesis. The amount of MT-2 on hepatocyes is acutely increased in iron deficiency. Hepcidin transcription is increased in the presence of inflammation. The cytokine interleukin-6 (IL-6) binds to the IL-6 membrane receptor. This activates the receptor and signals hepcidin transcription through the JAK-STAT2 cell signaling pathway.

- **HFE** is a transmembrane protein found primarily in hepatocytes, hematopoietic cells, and crypt cells of the duodenum. HFE can bind to both TfR1 and TfR2 on cells.[12] In the basal state of iron metabolism, HFE and TfR1 exist as a complex on the cell membrane. Diferric transferrin and HFE compete for binding to TfR1. When the level of diferric transferrin increases, as in iron-loading conditions, it displaces HFE from TfR1. The free HFE can then interact with TfR2, which can also bind transferrin. TfR1 and TfR2 communicate with one another through HFE. HFE complexed with TfR2 and transferrin act as iron sensors and convey the diferric-transferrin status through a cell signaling cascade, leading to hepcidin synthesis.[5,12,13] The signaling complex involves the bone morphogenic protein (BMP) receptor and a membrane anchored coreceptor, HJV, which together bind the BMP6 ligand and activate the BMP receptor.[14] Thus, HFE is involved in regulating iron absorption and uptake by modulating hepcidin levels. Mutations of HFE and TfR2 are associated with hereditary hemochromatosis (a condition of total body iron overload).[15]
- **Hemojuvelin (HJV)** is a glycosylphosphatidylinositol-anchored membrane-bound BMP coreceptor that enhances hepcidin gene expression.[5,6] Matriptase-2 (MT-2, *TMPRSS6*), which cleaves HJV, may modulate the HJV concentration on the cell membrane. HJV mutations are associated with decreased hepcidin synthesis and severe iron overload. However, the exact molecular mechanism is not yet known.

Thus, although hepcidin is the major iron-regulating hormone, additional proteins are involved in iron homeostasis by their influence on hepcidin synthesis or function.

Iron Balance at the Cellular Level

Control of iron balance at the cellular level occurs by regulation of transcription and translation of proteins involved in iron metabolism. Iron-regulatory proteins (IRPs) have mRNA with similar RNA stem-loop-stem structures in either the 5′ or 3′ noncoding regions, referred to as *iron-responsive elements (IREs)*. IREs are recognized and bound by IRPs. The binding affinity of IRP for the IRE is determined by the amount of cellular iron: IRP binds to the IRE when iron is scarce and dissociates when iron is plentiful.

When bound to the IRE of mRNA, the IRP modulates the translation of the mRNA. The IRP regulates translation in one of two ways, depending on the location of the IRE in the mRNA. IRP binding to IRE in the 5′ untranslated region (UTR) of the mRNA results in the disruption of translation by preventing the assembly of initiation factors at the initiator site (Figure 12-7 ■). Because binding occurs when iron is scarce, translation of proteins with IRE in the 5′ UTR is decreased in the absence of iron.[17–19] The mRNAs of ferritin, ferroportin, and ALA synthase 2 (ALAS2) fall into this group. If the IRE is in the 3′ UTR of the mRNA, binding of the IRP stabilizes the mRNA that would otherwise be digested/degraded (Figure 12-8 ■). Thus, translation increases when iron is scarce. The mRNAs of TfR and DMT1 fall into this second group.

Thus, IRP regulates cellular iron by coordinating synthesis of ferritin and TfR in opposite directions. The level of TfR expression reflects the cell's need for iron and is an important factor in hemoglobin synthesis. When the cell needs more iron, TfRs increase to maximize the amount of iron incorporated into the cell and ferritin formation decreases. On the other hand, when cells have adequate or excess iron, the ferritin levels rise and transferrin receptors decrease.

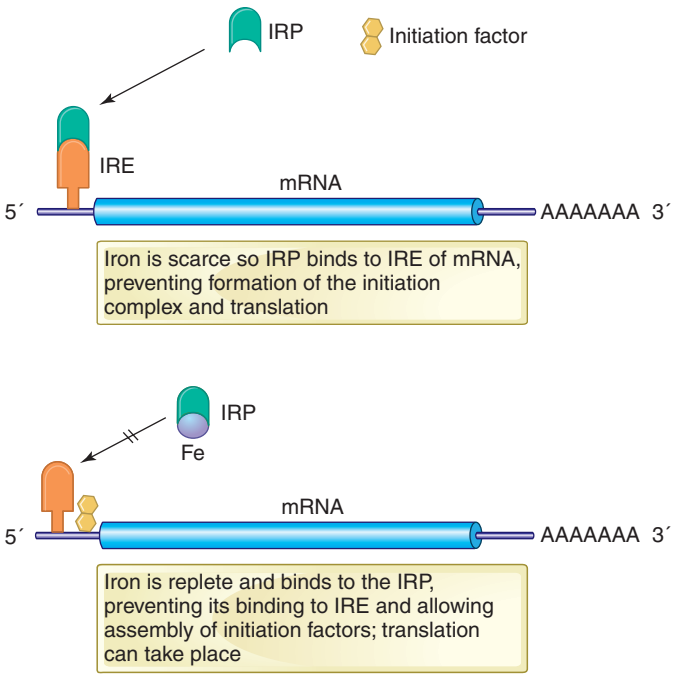


FIGURE 12-7 Regulation of iron at the cellular level. A stem-loop-stem structure in the 5′ noncoding region, referred to as the iron-responsive element (IRE), is present in some mRNA. Binding of iron-regulatory protein (IRP) to the IRE prevents initiation of translation. When the level of cellular iron is replete, the iron binds to IRP, preventing IRP binding to the IRE and allowing assembly of initiation factors at the initiation site, which allows translation to take place. The translation of mRNAs of ferritin, ferroportin, and ALAS2 is regulated in this fashion. (AAAAAAA = POLY-ADENINE TAIL)

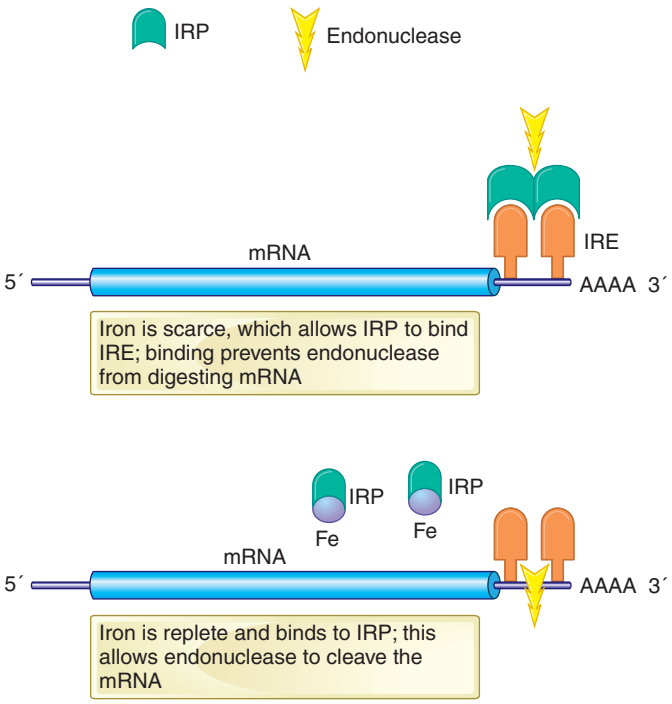


FIGURE 12-8 Regulation of iron at the cellular level. A stem-loop-stem structure in the 3′ noncoding region, referred to as the iron-responsive element (IRE), is present in some mRNA. Binding of iron-regulatory protein (IRP) to the IRE stabilizes the mRNA. When cellular iron is replete, the iron binds to the IRP and prevents it from binding to the IRE. This results in degradation of the mRNA by endonuclease. The translation of the mRNAs of TfR and DMT1 is regulated in this fashion. (AAAA = POLY-ADENINE TAIL)

Regulation of the ALAS2 gene coordinates the synthesis of porphyrin (heme) with iron availability.

☑ CHECKPOINT 12-1

In iron-deficiency anemia, would you expect synthesis of ALAS2 to increase or decrease? Explain.

Iron Metabolism in the Mitochondria

Most iron entering erythroid cells is routed to the mitochondria for hemoglobin synthesis and iron-sulfur (Fe-S) cluster (iron containing functional group) assembly. Mitochondrial ferritin serves as an iron storage molecule and is highly expressed in tissue with numerous mitochondria. It also is highly expressed in the sideroblasts of patients with X-linked sideroblastic anemia with ataxia (XLSA/A).

Frataxin is the protein in the mitochondrial matrix that is thought to play a role in mitochondrial iron export and storage.[20]

Iron Requirements

Normally, humans maintain a relatively constant body concentration of iron throughout their life. This is accomplished by establishing a positive iron balance during growing years and maintaining an equilibrium between loss and absorption in adult life. Humans are unable to excrete iron; therefore, the rate of absorption and loss of iron must be matched to avoid ID or excess.

Factors That Increase Fe Requirements

Normal physiologic factors that increase the daily requirement for iron include menstruation, pregnancy, and growth. Pathologic factors that increase the need for iron are discussed in the next section on iron-deficiency anemia.

Menstruation

The average daily iron loss in menstruating females is 0.6–2.5% more than in men and nonmenstruating women.[21] To maintain total body iron balance, menstruating females must absorb about 2 mg of iron daily.

Pregnancy

The daily iron requirement during pregnancy is about 3.4 mg; if spread out as a daily average over the 3 trimesters, it would be about 1000 mg per pregnancy. The fetus accumulates ~250 mg of iron from maternal stores via the placenta; added to this is the iron requirement for increased maternal blood volume and iron loss at delivery due to bleeding. Thus, a single pregnancy without supplemental iron could exhaust the mother's iron stores.

Infancy/Children

In infancy, rapid growth of body size and hemoglobin mass requires more iron in proportion to food intake than at any other time of life. During the first 6 months of life, an infant synthesizes ~50 g of new hemoglobin. In addition, iron is needed for tissue growth. At birth, normal iron stores of 30 mg are adequate to see the infant through the first 4 to 5 months of life but can be depleted quickly in an infant whose primary dietary intake is unfortified cow's milk. Extraction of iron from human breast milk is very efficient, enabling infants to acquire a large proportion of the iron present. Premature infants are at an even higher risk of rapid iron depletion because much of the placental transfer of iron occurs in the last trimester of pregnancy, and they have a faster rate of postnatal growth than full-term infants. In one study the prevalence of iron deficiency at 1 year corrected age of preterm very-low-birth-weight infants was 45%.[22] It is recommended that full-term infants begin iron supplements no later than 4 months of age and that low-birth-weight infants begin no later than 2 months of age.[23]

Iron requirements are also high in childhood, especially in 1- to 2-year-olds. Globally, about 25% of preschool children have iron-deficiency anemia.[24]

LABORATORY ASSESSMENT OF IRON

Clinically useful indicators of iron status change sequentially as body iron changes from replete to deficiency or overload. Additionally, coexisting conditions can affect test results. Because no single indicator or combination of indicators reveals true body iron status in all circumstances, it is recommended that multiple parameters be used to assess iron status.[25] Laboratory testing to determine iron status includes measurement of serum iron, total iron-binding capacity (TIBC), calculation of the percent saturation of transferrin, serum ferritin, and serum transferrin receptor (sTfR) (for reference intervals see Table F, back cover). An indirect assessment of iron availability is provided by the zinc protoporphyrin (ZPP) assay.

Iron Studies

Transferrin can be measured as a protein by immunochemical methods, but because the percent saturation (with iron) is helpful in the differential diagnosis of anemia, it is usually measured functionally as the maximum amount of iron able to be bound in the serum (TIBC). The measured serum iron and TIBC are used to calculate the percent saturation.

As a general rule, changes in the quantity of total body storage iron are accompanied by fluctuations in the serum iron and TIBC. As storage iron increases, serum iron increases and TIBC decreases; conversely, if storage iron decreases or is absent, serum iron decreases and TIBC increases. A transferrin saturation below 15% is an indicator of ID, whereas a saturation above 50% suggests iron overload and possibly hemochromatosis.

Ferritin can be measured in the serum, where its concentration is proportional to the amount of storage iron in the body. Generally, serum ferritin levels <12 mcg/L indicate depletion of iron stores while levels >1000 mcg/L indicate iron overload. Decreased serum ferritin levels can be the first indication of developing IDA. Serum ferritin levels decrease before the exhaustion of mobilizable iron stores, whereas abnormalities in the TIBC and serum iron may become detectable only after iron stores are depleted. Ferritin is an acute phase reactant, so care should be used in interpreting serum ferritin levels in infectious or inflammatory states. Concomitant ID can be masked if other tests of iron status are not considered. Refer to Table 12-7 ★ for the variations in tissue iron in various disease states.

Small amounts of TfR can be identified in serum (sTfR) by sensitive immunoassay techniques. The level of circulating sTfR mirrors the amount of cellular receptor. The sTfR is inversely proportional to the amount of body iron because cellular receptor synthesis increases when cells lack sufficient iron. The majority of sTfR is derived from the erythroid cells in the bone marrow; the concentration is directly proportional to erythroid activity and parallels the reticulocyte count. The level of sTfR is not affected by concurrent disease states as is serum ferritin. Circulating sTfR increases in iron-deficiency anemia but not anemia of chronic disease and thus is useful in the differential diagnosis.

When iron is not available for incorporation into the protoporphyrin ring to form heme or heme synthesis is disturbed, zinc is an alternate protoporphyrin ligand and can be incorporated into the ring, forming zinc protoporphyrin (ZPP). As a result, excess protoporphyrin in the form of ZPP can accumulate in the cell. The ZPP formed during RBC development persists for the life of the cell and thus reflects iron supply over the preceding weeks. ZPP can be detected by measuring fluorescence in the blood.

☑ CHECKPOINT 12-2

A patient's iron studies revealed serum iron 100 mcg/dL and TIBC 360 mcg/dL. Calculate the percent saturation and UIBC. Are these values normal or abnormal?

★ **TABLE 12-7** Iron Status Parameters in Patients with Various Diseases

Disease	MCV	Serum iron	Saturation (%)	Ferritin	Serum Transferrin Receptor
Iron-deficiency anemia	Low	Low	Low	Low	Increased
Acute infection	Normal	Low	Low	Increased	Normal
Anemia of chronic disease	Normal or low	Low	Low to normal	Normal to Increased	Normal
Thalassemia minor	Low	Normal	Normal	Normal	Normal to increased
Hypoplastic/aplastic anemia	Normal to increased	High	High	Increased	Normal
Acute hepatitis	Normal	Increased	Normal to increased	Increased	Normal
Chronic liver disease	Normal to increased	Low to normal	Normal to increased	Increased	Normal

Complete Blood Count (CBC)

The normocytic, normochromic red blood cells are gradually replaced by microcytic, hypochromic cells in ID. The time it takes for abnormal cells to replace the normal population depends on the extent of the demand for iron and the amount available. The RDW increases as the microcytic cells replace the normocytic cells. Some hematology analyzers give the proportion of hypochromic cells. Analyzers may also give the mean cellular hemoglobin of reticulocytes (CHr, MCHr) or reticulocyte hemoglobin equivalent (CHret) (Chapter 11). The CHr measures the functional availability of iron during hemoglobin synthesis in the erythrocyte. It provides an early indication of iron-restricted erythropoiesis before anemia develops.

Hepcidin

Hepcidin is the primary controller of iron supply and storage because of its effect on iron absorption and ferroportin1. Although hepcidin measurement has the potential to increase diagnostic accuracy of iron-metabolism disorders, a simple, inexpensive, widely available test has not been developed.

Ferrokinetics

Quantitative measurement of internal iron exchange (ferrokinetics) is useful in understanding the pathophysiology of certain erythropoietic disorders. Ferrokinetic studies monitor the movement of radioactively labeled iron (^{59}Fe) from the plasma to the bone marrow and its subsequent uptake into developing erythroblasts. Plasma iron is labeled by intravenous injection of a trace amount of ^{59}Fe. The labeled iron binds to transferrin for transport. Its clearance from the plasma can be followed by counting the radioactivity that remains in the plasma at intervals up to 90 minutes. The rate at which iron leaves the plasma is called the *plasma iron turnover* (*PIT*) rate, and its primary determinant is tissue need. The PIT is a good indicator of total erythropoiesis and correlates well with the erythroid cellularity of bone marrow.

The amount of iron used for effective hemoglobin synthesis can also be measured by determining the amount of ^{59}Fe incorporated into circulating erythrocytes over time. Normal erythrocyte utilization is 70–90% of the injected ^{59}Fe by day 10–14. This is termed the *erythrocyte iron turnover (EIT)* rate. The EIT is a good measure of effective erythropoiesis and correlates with the reticulocyte production index.

The normal discrepancy between the rate at which iron leaves the plasma (PIT = 0.7 mg/day/dL) and the rate at which it moves from marrow to circulating erythrocytes (EIT = 0.56 mg/day/dL) suggests that the red cell utilization (RCU) of iron is <100%. Some of the labeled iron can enter the liver or bone marrow macrophages. In addition, 5–10% of bone marrow iron is involved in ineffective erythropoiesis, causing a loss of the labeled iron by intramedullary destruction of abnormal erythrocytes.

A rapid or increased PIT coupled with a normal or increased RCU indicates increased erythropoiesis. A normal to increased PIT coupled with a decreased RCU indicates ineffective erythropoiesis, and a decreased PIT with corresponding decreased RCU indicates decreased erythropoiesis.

Ferrokinetic studies can be valuable in locating sites of medullary and extramedullary erythropoiesis by counting surface radioactivity over the liver, spleen, and sacrum.

IRON-DEFICIENCY ANEMIA

Iron deficiency is the most common nutritional deficiency in the world. It is prevalent in countries where grain is the mainstay of the diet or meat is scarce. Unfortunately, these are generally countries where hookworm infestation is endemic. The combination of decreased availability of dietary iron and chronic blood loss from parasitic infection increases the risk of developing IDA. Malnutrition is associated not only with decreased iron intake but also with decreased intake of other essential nutrients including folate. Thus, causes of anemia associated with malnutrition can be multifactorial.

Historical Aspects

In the United States between 1870 and 1920, *chlorosis*, a word used to describe the condition of ID, was so common in young women that it was believed that every female had some form of the disease during puberty.[26] The word *chlorosis* was coined because of the greenish tinge of the skin in these patients; however, often the greenish hue was not apparent but pallor (paleness of the skin) was pronounced. Other classic clinical signs and symptoms of anemia were present including shortness of breath on exertion, lethargy, and heart palpitations. These chlorotic girls were found to have decreased numbers of red cells and an increase in the proportion of serum to cells in their blood. Some of the chlorotic girls were also noted to have unusual appetites, craving substances such as chalk, cinders, charcoal, and bugs. As therapy for these patients, doctors prescribed iron salts even though the exact nature of the disease had not been identified. Some physicians linked chlorosis to dietary habits; others implicated menstruation as a possible cause of the disease because chlorosis affected girls in puberty but not boys.

Etiology

ID can occur because of normal or pathologic conditions that result in an increased demand for iron, malabsorption, or poor diet. In malabsorption or with an iron-deficient diet, iron stores can become depleted over a period of years. With an increase in the demand for iron, iron depletion can occur more rapidly, sometimes over a period of months.

Dietary Deficiency

In most developed countries, inadequate dietary intake of iron is rarely the cause of anemia (except in infancy, pregnancy, and adolescence). Diet and socioeconomic status, however, are factors in the development of iron deficiency in children.

Blood Loss

The average adult male in the United States ingests many times more iron than is required. It would take an adult male about 8 years to develop IDA if he absorbed no iron during that period of time. ID in adult males is almost always due to chronic blood loss from the gastrointestinal or genitourinary tracts. For each milliliter of blood lost, a loss of about 0.5 mg of iron occurs. Gastrointestinal lesions leading to blood loss include peptic ulcers, hiatal hernia, malignancies, alcoholic gastritis, excessive salicylate ingestion, hookworm infestation, and hemorrhoids. The correlation of ID and GI lesions in elderly patients is high.[27] Genitourinary tract blood loss occurs less frequently and can result from lesions within the genitourinary system.

In women of child-bearing age, iron loss through menstruation is the most frequent cause of iron deficiency.[28] Blood loss is less often the result of intravascular hemolysis. If haptoglobin becomes depleted, the free circulating hemoglobin dissociates into dimers, is filtered by the kidneys, and appears in the urine (hemoglobinuria). This results in a loss of iron, the amount of which is proportional to the amount of hemoglobin in the urine. Some of the hemoglobin is reabsorbed in the renal tubules, resulting in the deposition of iron as hemosiderin in renal tubular cells and eventual sloughing of these cells into the urine (hemosiderinuria).

Hemodialysis

Anemia is a common finding in patients with kidney disease. The causes are varied, but the most important factor is a loss or decrease in erythropoietin (EPO) production by the diseased kidneys. Thus, hemodialysis patients are often given recombinant human erythropoietin (rHuEPO) to increase erythropoiesis. However, sufficient iron cannot be mobilized from storage sites rapidly enough to meet the need for a two- to four-fold increase in the rate of erythropoiesis that can result from this therapy. The result can be a functional iron deficiency.[29] Lack of an adequate response to EPO therapy is primarily due to functional ID.[30] However, if the patient is given intravenous iron injections together with rHuEPO, the hematopoietic response is enhanced.

Malabsorption

Malabsorption is an uncommon cause of ID except in malabsorption syndromes (such as sprue), after gastrectomy, in atrophic gastritis, and in achlorhydria. Gastrectomy results in impaired iron absorption due to the absence of gastric juice, which helps to solubilize and reduce dietary iron into the more easily absorbed ferrous form. In addition, with the loss of the reservoir function of the stomach, nutrients can transit rapidly through the duodenum, allowing insufficient time for iron absorption.

☑ CHECKPOINT 12-3

A 30-year-old female and a 25-year-old male both had bleeding ulcers. Assume that they acquired the ulcer at the same time, were losing about the same amount of blood, had equal amounts of storage iron to begin with, and were taking in about 15 mg of dietary iron each day. Would you expect that the woman and man would develop ID at the same time? Explain.

IDA is frequently accompanied by atrophic gastritis and achlorhydria, but it is not known whether achlorhydria and gastritis are causes of iron malabsorption or the ID is a cause of atrophic gastritis and hence achlorhydria. In patients with gastritis and achlorhydria, therapy with oral iron can be ineffective due to poor iron absorption.

Pathophysiology

Defined as a diminished total body iron content, ID develops in sequential stages during a period of negative iron balance (losing more iron than is absorbed in the gut). These stages are commonly referred to as:

- Iron depletion
- Iron-deficient erythropoiesis
- IDA

Thus, ID can range in severity from reduced iron stores with no functional effect (Stages 1 and 2) to severe anemia with deficiencies of tissue iron-containing enzymes (Stage 3). Laboratory evaluation of iron status is helpful in defining these 3 stages (Table 12-8 ★).

- *Stage 1* During iron depletion, iron stores are exhausted as indicated by a decrease in serum ferritin. There is no anemia and erythrocyte morphology is normal, but the red cell distribution width (RDW) is frequently elevated. The abnormal RDW can be the first hematologic indication of a developing ID in the nonanemic patient.

  In hospitalized patients, the RDW is not as specific, and the serum ferritin not as sensitive in detecting ID.[31] Hospitalized patients have a high incidence of other diseases that can affect these parameters.

- *Stage 2* The second stage of ID is characterized by iron-deficient erythropoiesis. There is insufficient iron to insert into the protoporphyrin ring to form heme. As a result, the protoporphyrin accumulates in the cell and complexes with zinc to form ZPP. Bone marrow sideroblasts are absent; macrophage iron is not seen.

  Anemia and hypochromia are still not detectable, but the erythrocytes can become slightly microcytic. The CHr, which measures the functional availability of iron during hemoglobin synthesis, can decrease even when anemia is absent (Chapter 11).[32]

- *Stage 3* A long-standing negative iron flow eventually leads to the last stage of iron deficiency: IDA. Blood loss can significantly shorten the time for this stage to develop. All laboratory tests for iron status become markedly abnormal. The most significant finding is the classic microcytic hypochromic anemia.

It is apparent, then, that when microcytic hypochromic anemia due to ID is present, the situation represents the advanced stage of severely deficient total body iron.

Clinical Features

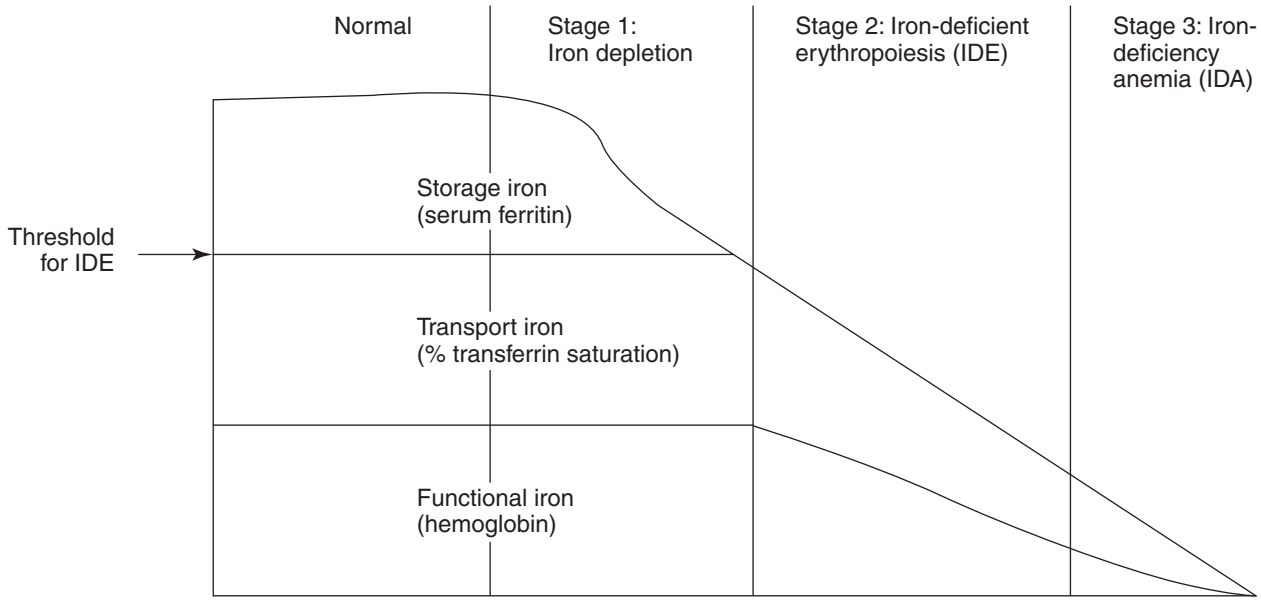
The onset of IDA is insidious, usually occurring over a period of months to years. Early ID stages usually show no clinical manifestations, but as anemia develops, clinical symptoms appear. In addition to symptoms of anemia, a variety of other abnormalities can occur due to a decrease or absence of iron-containing enzymes in various tissues. These include koilonychia (concavity of nails), glossitis, pharyngeal webs, muscle dysfunction, inability to regulate body temperature when cold or stressed, and gastritis.

A curious manifestation of ID is the *pica* syndrome. **Pica** is an unusual craving for ingesting unnatural items. The most common dysphagias described in patients with ID include ice-eating (phagophagia), dirt/clay-eating (geophagia), and starch-eating (amylophagia).[33,34]

Iron-deficient infants perform worse in tests of mental and motor development than do nonanemic infants.[35] There is speculation that untreated ID at this stage of human development has long-lasting effects on the central nervous system.[36] Symptoms reported to occur in iron-deficient children include irritability, loss of memory, and difficulties learning. Deficiencies of the immune system have been attributed to iron-related impairment of host defense mechanisms.

In the absence of iron in the gut, other metals are absorbed in increased amounts. This can be significant when an iron-deficient person is exposed to toxic metals such as lead, cadmium, and plutonium.

★ **TABLE 12-8** Laboratory Test Profile of Fe Status in Developing Iron Deficiency



Parameter	Reference Interval	Stage 1 Storage Iron Depletion; No Anemia	Stage 2 IDE	Stage 3 IDA
Ferritin (mcg/L)	Male 20–300 Female 12–200	Decreased	Decreased	Decreased
sTfR (mg/L)	1.15–2.75	Normal	Increased	Increased
TfR-F Index	0.63–1.80	Increased	Increased	Increased
Serum iron (mcg/dL)	Male 65–180 Female 50–180	Normal	Decreased	Decreased
TIBC (mcg/dL)	250–450	Normal or slight increase	Increased	Increased
saturation %	Male 20–45 Female 15–45	Normal	Decreased	Decreased
ZPP (mcg/dL) whole blood corrected to volume of packed red cells of 0.35 L/L	Male 1–27 Female 11–45	Increased	Increased	Increased
Male hemoglobin (g/L)	140–174	Normal	Normal	Decreased
Female hemoglobin (g/L)	120–160	Normal	Normal	Decreased
RBC morphology	Normocytic, normochromic	Normocytic, normochromic	Normocytic, normochromic	Microcytic, hypochromic

sTfR = serum transferrin receptor; TfR-F = transferrin receptor-ferritin; TIBC = total iron binding capacity; ZPP = zinc protoporphyrin

Laboratory Features

Practice guidelines for diagnosis and therapy of iron deficiency are inconsistent.[26] However, laboratory tests are essential for an accurate diagnosis and in evaluation of therapy.

Peripheral Blood

The blood picture in well-developed ID is microcytic (MCV 55–74 fL) and hypochromic (MCHC 22–31 g/dL, MCH 14–26 pg) (Figure 12-9 ■). Because ID develops progressively, any gradation between the well-developed microcytic hypochromic iron-deficient blood picture and normal can occur. Microcytosis and anisocytosis, characterized by an increased RDW, are usually the first morphologic signs, developing even before anemia (Chapter 11). The blood film demonstrates progressive poikilocytosis. The most frequent poikilocytes are target cells (codocytes), elliptocytes, and dacryocytes (teardrop cells).

The typical blood picture can be masked if the iron-deficient patient has a concurrent vitamin B_{12} or folate deficiency (causes of macrocytic anemia). In these cases, microcytosis may become apparent only after vitamin B_{12}/folic acid replacement therapy.

Both the relative and absolute number of reticulocytes can be normal or even slightly increased, but the reticulocyte count is decreased relative to the severity of the anemia with an RPI of <2. A decreased CHr is an early indicator of iron-restricted erythropoiesis in nonanemic individuals. In patients on chronic hemodialysis, the CHr has been shown to be superior to the presence of hypochromic

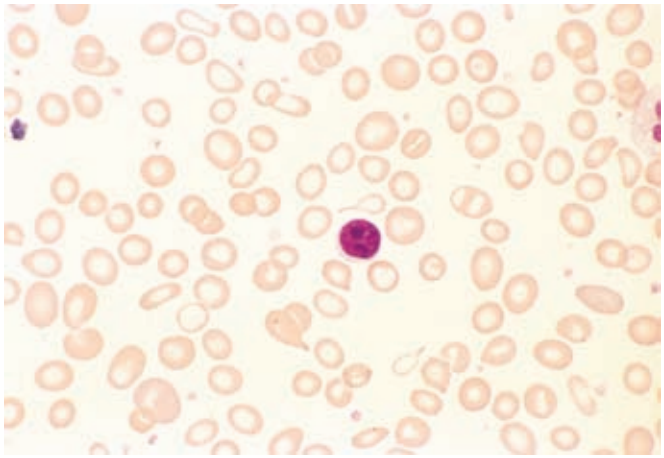


FIGURE 12-9 Microcytic, hypochromic anemia of iron deficiency. Compare size of RBCs to nucleus of small lymphocyte in center. Note the anisocytosis and poikilocytosis (peripheral blood; Wright-Giemsa stain; 1000× magnification).

red cells in detecting iron deficiency. Using a cutoff of CHr <26, the CHr has a sensitivity of 100% and a specificity of 73%.[37] In comparison, the sensitivity and specificity in detecting iron deficiency when the percentage of hypochromic red cells is >2.5% is 91% and 54% respectively.

The leukocyte count is usually normal but can increase because of chronic marrow stimulation in long-standing cases or after hemorrhage. With concomitant hookworm infestation, eosinophilia can be present.

Platelets can be normal, increased, or decreased. Thrombocytosis frequently accompanies ID, and has been proposed to be related to ID caused by chronic blood loss. Thrombocytopenia can occur in patients with severe or long-standing anemia, especially if accompanied by folate deficiency. Platelet numeric abnormalities can be corrected with treatment that replenishes iron stores.

CASE STUDY *(continued from page 199)*

Jose, the 83-year-old patient, had a CBC upon admission. The results were:

RBC	4.15×10^{12}/L
Hb	81 g/L (8.1 g/dL)
Hct	0.26 L/L (26%)
Platelets	174×10^{9}/L
WBC	2.8×10^{9}/L

2. How would you describe his anemia morphologically?

Iron Studies

Iron studies on iron-deficient patients help establish the diagnosis. The serum iron is decreased, usually less than 30 mcg/dL, the TIBC is increased, and transferrin saturation is decreased to less than 15%. Serum iron concentration has a diurnal variation with highest levels in the morning, so sampling time is an important consideration.

The serum ferritin level is decreased in all stages of ID and can be the first indication of a developing ID state. Serum ferritin is generally considered the single best test to detect iron deficiency. Once serum ferritin levels fall below 12 mcg/L, the levels no longer correlate with storage iron because stores are exhausted. Serum ferritin is an important test to differentiate IDA from other microcytic hypochromic anemias. Levels are normal to increased in ACD unless complicated by ID and increased in sideroblastic anemia and thalassemia.

Because serum ferritin is an acute phase reactant, the lower limit of the reference interval for this parameter could need to be adjusted to detect ID in some patient populations (Web Table 12-1). It has been suggested that to detect ID in patients with concomitant ACD, inflammation, infection, pregnancy, and a wide range of other medical problems, the lower limit of serum ferritin should be raised from 12 g/L.[30,38–41] It has also been suggested that the threshold level of serum ferritin for a diagnosis of ID in the aged subject be raised because serum ferritin levels rise with age.[42]

The sTfR assay has proved useful in detecting and differentiating IDA and ACD. Patients with ID have a mean sTfR level over twice (13.91 + 4.63 mg/L) that of normal individuals (5.36 + 0.82 mg/L). Conversely, patients with chronic disease and acute infection (ACD) have mean levels almost identical to those of normal individuals. A combination of sTfR and serum ferritin can be used to calculate the sTfR-F index:

$$\text{sTfR-F Index} = \text{sTfR/log ferritin}$$

This index can improve detection of subclinical iron-deficient states in healthy individuals.[43,44] An index of more than 1.8 indicates depletion of iron stores.

Combinations of serum ferritin, MCV, TIBC, percent saturation, and sTfR can eliminate the need for costly, inconvenient, and painful bone marrow examination to assess iron stores in patients with inflammation or chronic disease and in early stages of ID.[38,45,46]

Erythrocyte Protoporphyrin (EP) Studies

The ZPP measurement correlates inversely with serum ferritin concentration and is more cost effective.[47] It can detect iron depletion before anemia develops and thus is a good screening tool for the early stages of ID. Because the number of nonanemic toddlers with iron depletion is significant and ID has a detrimental effect on development in children, ZPP rather than the hematocrit should be utilized as the screening test for ID.[48] ZPP is also more diagnostically efficient than serum iron or serum ferritin in screening for ID in the presence of infection or inflammation and in hospitalized patients with microcytic anemia.[47,49] Thus, ZPP can be a valuable screening assay for these populations.

The level of ZPP is useful also as a screening test to differentiate ID and thalassemia, the two most common causes of microcytic hypochromic anemia. Whereas ZPP can be elevated in thalassemia, the increase in ID is 3 to 4 times higher than in thalasssemia.[50] When laboratory analysis reveals a high ZPP combined with a high RDW, ID is strongly suggested.[51] The RDW is typically normal or only slightly elevated in thalassemia trait. Web Table 12-2 lists other conditions associated with increased EP levels.

In patients with concurrent lead poisoning, the ZPP cannot be used to distinguish ID and thalassemia because lead inhibits

CASE STUDY (continued from page 213)

Reflex testing for anemia on Jose followed based on the CBC results. The following test results were obtained:

Reticulocyte count	2.6%
Serum iron	18 mcg/dL
TIBC	425 mcg/dL

3. Calculate percent saturation.
4. Is this value normal, decreased, or increased?
5. What disease, if any, does this value suggest?

ferrochelatase, the enzyme needed to incorporate iron into the protoporphyrin ring. Consequently, the free erythrocyte porphyrin complexes with zinc, and ZPP is increased in lead poisoning whether or not iron is available.

Bone Marrow

The bone marrow shows mild to moderate erythroid hyperplasia with a decreased M:E ratio. Total cellularity is often moderately increased. This increase in marrow erythropoietic activity without a corresponding increase in peripheral blood reticulocytes suggests an ineffective erythropoietic component. With appropriate iron therapy, the erythroid hyperplasia initially increases and then returns to normal. A common finding (not exclusive to IDA) is the presence of poorly hemoglobinized erythroblasts with scanty irregular (ragged) cytoplasm. This morphology is most evident at the polychromatophilic stage. Erythroid nuclear abnormalities are sometimes present and can resemble the changes found in dyserythropoietic anemia. These changes include budding, karyorrhexis, nuclear fragmentation, and multinuclearity. Stains for iron reveal an absence of hemosiderin in the macrophages, a consistent finding in ID. Sideroblasts are markedly reduced or absent. Evaluation of iron stores using serum iron studies eliminates the need for bone marrow examination in most cases.

Therapy

Once the cause of the anemia has been established, treatment principles are to address the underlying disorder (e.g., bleeding ulcer), administer iron, and observe the response. The anemia is usually corrected by the oral administration of ferrous sulfate. Parenteral iron therapy, which is more dangerous and expensive than oral iron therapy, is indicated only rarely for unusual circumstances. Intravenous iron dextran is often required in patients with chronic renal disease who are receiving therapy with rHuEPO.[52] To maintain a two-fold to three-fold increase in the rate of erythrocyte production in patients treated with rHuEPO, enough iron should be given to maintain serum iron concentrations at 80–100 mcg/dL.[53]

Iron-deficient patients treated with iron experience a return of strength, appetite, and a feeling of well-being within 3–5 days, whereas the anemia is not alleviated for weeks. The dysphagias also are corrected before the anemia.

A response to iron therapy is defined as an increase of 1 gm hemoglobin in 1 month. Because reticulocytes are new red blood cells just released from the bone marrow, reticulocyte counts and IRF give a snapshot of recent red blood cell production (Chapter 11). Reticulocyte response to iron therapy begins about the 3rd day after the start of therapy, peaks at about the 9th to 10th day (4–10% reticulocytes), and declines thereafter. An increase in CHr (Chapter 11) is an early indicator of the availability of functional iron and its incorporation into hemoglobin over the last several days. The CHr begins to increase well in advance of an increase in reticulocytes and hemoglobin.[37,54] If therapy is successful, the hemoglobin should rise until levels within reference intervals are established, usually within 6–10 weeks. To restore iron stores, extended therapy with small amounts of iron salts may be required (usually for 6 months) after the hemoglobin has returned to normal.

Because of the high prevalence of ID in toddlers, screening all 1-year olds for iron deficiency and/or anemia is a well-accepted practice. It has been suggested that if the hemoglobin concentration is decreased, a therapeutic trial of iron be initiated in this population. If anemia persists after 1 month of therapy, further evaluation is necessary[55] (Web Figure 12-1).

ANEMIA OF CHRONIC DISEASE

ACD, also called *anemia of inflammation* or *infection (AI)* is usually defined as the anemia that occurs in patients with chronic infections, chronic inflammatory disorders, trauma, organ failure, or neoplastic disorders not due to bleeding, hemolysis, or marrow involvement (Table 12-9 ★). ACD is characterized by low serum iron but normal iron stores. The anemia appears to be a specific entity and does not relate to any nutritional deficiency. Anemias associated with renal, endocrine, or hepatic insufficiency are usually excluded from ACD.

ACD is the most common anemia other than IDA. It accounts for the anemia in more than one-third of anemic hospitalized patients without blood loss. Anemia is present in up to 50% of patients with malignant solid tumors and is often the clue that leads to a diagnosis of cancer. The most common anemia in these patients is ACD.

Pathophysiology

The causes of ACD are multifactorial (Table 12-10 ★). Most of the pathophysiology is linked to inflammatory cytokines. ACD is characterized by hypoferremia, decreased transferrin (decreased TIBC), increased serum ferritin, and increased iron in bone marrow macrophages. This suggests a block in the mobilization of iron from macrophages for recycling to the bone marrow erythroblasts. Because macrophages recycle 10–20 times more iron than is absorbed by enterocytes, any changes in iron flux through the macrophages affect iron balance more rapidly than changes in iron absorption and transport by enterocytes. Absorption of iron in the intestine is decreased, which can lead to iron deficiency over time.

Studies reveal that the block in iron release from macrophages is mediated by hepcidin, which is produced in response to interleukin 6 (IL-6)

★ **TABLE 12-9** Conditions Associated with Anemia of Chronic Disorders

Category	Conditions
Chronic infections (after 1–2 months of sustained infection)	Pulmonary infections Subacute bacterial endocarditis Pelvic inflammatory disease Osteomyelitis Chronic urinary tract infection Chronic fungal disease Tuberculosis
Chronic, noninfectious inflammation	Rheumatoid arthritis Rheumatic fever Systemic lupus erythematosus Sterile abscess Regional enteritis Ulcerative colitis
Other	Malignant diseases Alcoholic liver disease Congestive heart failure Thrombophlebitis Ischemic heart disease Severe tissue trauma Thermal injury Fractures

and other inflammatory cytokines found in ACD (Web Figure 12-2). These cytokines upregulate the transcription factors STAT3 and SMAD, which activate the hepcidin gene promoter. Hepcidin plays a role in the body's innate immune system, which is designed to sequester iron from pathogens and thus restrict their growth.

Other contributing factors to this anemia are decreased erythropoietin (EPO) production and direct suppression of erythropoiesis by cytokines[56,57] (Table 12-10). In general, the EPO levels in patients with anemia of chronic disease are lower than in anemic patients with similar hemoglobin levels but without chronic disease.[58,59] This finding suggests a blunted EPO response to anemia in ACD. Pharmacologic doses of EPO overcome this inhibitory effect and correct the anemia in some patients.[60]

ACD is also associated with shortened erythrocyte survival as a result of extracorpuscular factors. The reason for the decreased erythrocyte life span is unknown, but several mechanisms have been suggested including nonspecific macrophage activation, hemolytic factors elaborated by tumors, vascular factors, and the presence of bacterial toxins capable of hemolyzing erythrocytes. Of all mechanisms described, the block in release of iron from macrophages appears to be the most significant in the pathogenesis of ACD.

★ **TABLE 12-10** Mechanisms of Anemia in Anemia of Chronic Disease

- Block in release of iron from macrophages due to increased hepcidin
- Cytokine inhibition of EPO production
- Direct cytokine inhibition of erythropoiesis
- Shortened erythrocyte survival

Clinical Features

The signs and symptoms of ACD are usually those associated with the underlying disorder. Rarely severe, the degree of the anemia roughly correlates with the activity of the underlying disease.

Laboratory Features

Many of the laboratory test results are nonspecific in ACD, but with clinical signs and primary diagnosis, they help to establish the ACD diagnosis. This is important because ACD does not usually require therapy for the anemia.

Peripheral Blood

A mild anemia with a hemoglobin of not <90 g/L and hematocrit of not <0.27 L/L is characteristic. Erythrocytes are usually normocytic (MCV >85 fL) and normochromic but can present as normocytic, hypochromic, or in long-standing cases, microcytic and hypochromic (Figure 12-10 ■). The reticulocyte production index is <2. The white blood cell count and platelet count are normal unless they are altered due to the primary disease state.

☑ CHECKPOINT 12-4

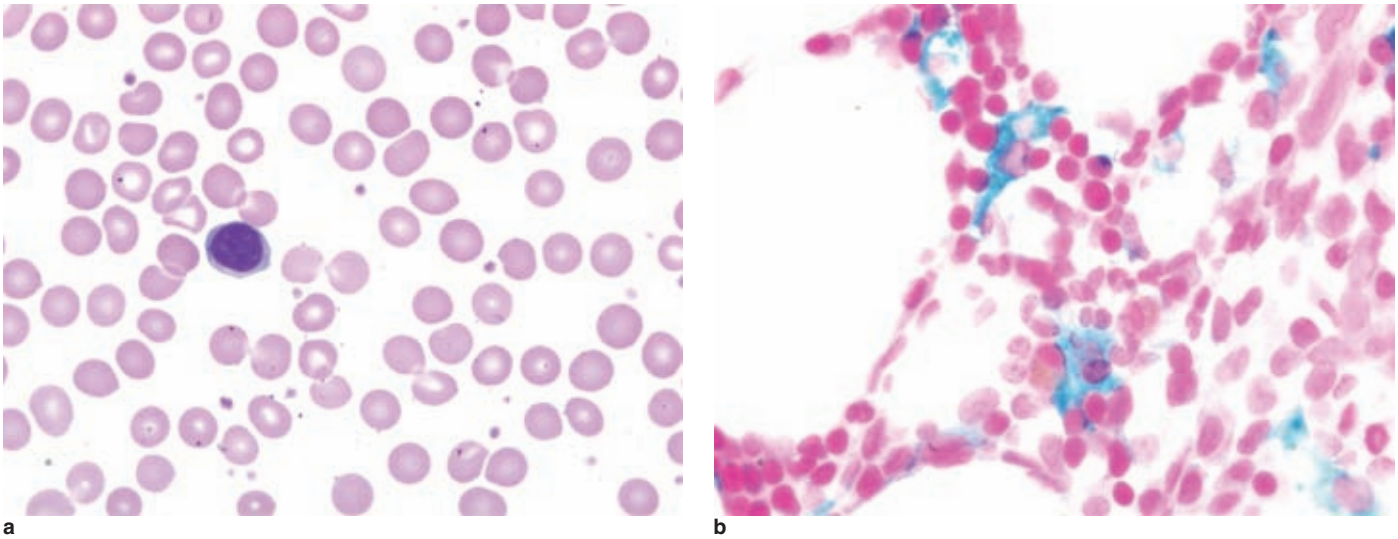
How does the peripheral blood picture in ACD differ from that seen in IDA?

Iron studies show decreased serum iron (10–70 mcg/dL), decreased to normal TIBC (100–300 mcg/dL), normal to low transferrin saturation (10–25%), and normal to increased serum ferritin. Serum ferritin can be helpful in distinguishing this anemia from IDA. Although serum iron is low in both anemias, serum ferritin, which reflects body iron stores, is normal or increased in ACD and low in IDA. Because ACD is characterized by iron-deficient erythropoiesis, the ZPP levels are increased.

It is important to remember that ferritin is an acute phase reactant and is very often increased in inflammatory conditions. Therefore, in ACD serum ferritin can be normal even if concurrent ID exists. If serum ferritin falls in the interval of 20–100 mcg/L in ACD, another means of assessing iron should be considered, such as sTfR assay, sTfR-ferritin index, or bone marrow examination.[61] Serum TfR is high in IDA but normal in uncomplicated ACD. The sTfR-ferritin index is increased in iron deficiency and decreased in ACD.

CASE STUDY *(continued from page 214)*

6. How do the patient's iron study results help in differentiating the diagnosis of iron deficiency from ACD?
7. What additional iron test that was not done would be most helpful in this case?



a **b**

■ **FIGURE 12-10** (**a**) Blood film from a patient with anemia of chronic disease. Hb 96 g/L; MCV 76 fL; MCH 24 pg; MCHC 30.6 g/dL. The erythrocytes appear microcytic (peripheral blood; Wright-Giemsa stain; 1000× magnification). (**b**) Bone marrow from same patient in (a) stained with Prussian blue. Note the macrophages with abundant blue staining iron (bone marrow; Prussian blue stain; 1000× magnification).

Bone Marrow

The bone marrow usually shows an increased M:E ratio because of a decrease in erythrocyte precursors. The proportion of younger erythroblasts increases. Poor hemoglobin production is apparent, especially in the polychromatophilic erythroblasts. The proportion of sideroblasts decreases to <30%; however, the macrophages appear to have increased amounts of hemosiderin in the form of coarse iron aggregates (Figure 12-10b). This finding helps to distinguish ACD from IDA. In IDA, macrophage iron and sideroblasts are absent.

Bone marrow examination is usually not necessary for distinguishing ACD from IDA. An algorithm using the MCV, serum ferritin, and iron saturation can correctly differentiate and classify most cases of ACD and IDA.[50]

Therapy

Anemia can be alleviated by successful treatment of the underlying disease. Anemia is usually mild and nonprogressive; thus, transfusion is rarely warranted except in older patients with vascular disease and circulatory insufficiency.

ANEMIAS ASSOCIATED WITH ABNORMAL HEME SYNTHESIS

These anemias are associated with defects in enzymes of the heme biosynthetic pathway leading to abnormal heme synthesis. Iron incorporation into the protoporphyrin ring to form heme can be blocked. In contrast to IDA, the positive iron balance in these anemias can lead to an increase in iron stores predominantly in the spleen, liver, and bone marrow. Serum ferritin levels > 250 mcg/L in the male and >200 mcg/L in the female indicate increased iron stores.

The conditions discussed in this section include sideroblastic anemia and the porphyrias. Lead poisoning also is included because of its pathophysiologic relationship to these anemias through a block in heme synthesis.

Sideroblastic Anemias

Mutations that affect the first enzymatic step in heme synthesis, the formation of ALA, result in *sideroblastic anemia* (Chapter 6). Mutations in the subsequent steps of heme synthesis result in metabolic disorders called *porphyrias*. Sideroblastic anemia (SA) is the result of diverse clinical and biochemical manifestations that reflect multiple underlying hereditary, congenital, or acquired pathogenic mechanisms. However, all types are characterized by (1) an increase in total body iron, (2) the presence of ring sideroblasts in the bone marrow, and (3) hypochromic anemia.

Classification

The classification of SA is arbitrary at best, and many different schemes of classification exist. The one in Table 12-11 ★ is among the most descriptive and separates those that are inherited and those that are acquired.

The most common form of hereditary SA is due to a defective X-linked recessive gene. Although carrier females often show a dimorphic population of both morphologically abnormal and

★ **TABLE 12-11** Classification of Sideroblastic Anemia

Hereditary	Acquired
• Sex linked • Autosomal recessive	• Idiopathic refractory sideroblastic anemia (IRSA) or refractory anemia with ring sideroblasts (RARS) • Secondary to drugs, toxins, lead • Associated with malignancy

normal erythrocytes, they rarely have anemia. Affected males demonstrate the typical SA findings. In rare instances, both sexes are equally affected, implying the presence of another hereditary form that is transmitted in an autosomal recessive manner. In the hereditary forms, anemia can become apparent in infancy but most commonly appears in young adulthood. Occasionally, symptoms do not occur until age 60.

The acquired forms of sideroblastic anemia are more common than the hereditary forms. The acquired forms are classified according to whether the basis of the anemia is unknown (idiopathic) or is secondary to an underlying disease or toxin (secondary type). The idiopathic form, *refractory anemia with ring sideroblasts* (*RARS*), can affect either sex in adult life. It is included in a group of acquired stem cell disorders called *myelodysplastic syndromes* (Chapter 25) that have a tendency to terminate in acute leukemia. The acquired secondary type SAs are associated with malignancy, drugs, or other toxic substances. In this type, once the underlying disorder is effectively treated or the toxin removed, the anemia abates.

Pathophysiology

Studies of patients with SA have shown disturbances of the enzymes regulating heme synthesis. Ring sideroblasts are specific findings for these heme enzyme abnormalities. Ring sideroblasts are formed from an accumulation of nonferritin iron in the mitochondria that encircle the erythroblast nucleus. The mitochondria eventually rupture as they become iron laden. When stained with Prussian blue, the iron appears as blue punctate deposits circling the nucleus (ring sideroblasts). Iron within the erythroblasts is normally deposited diffusely throughout the cytoplasm in siderosomes.

Hereditary Sideroblastic Anemia

The most common form of hereditary SA is sex linked and due to an abnormal δ-aminolevulinic acid synthase enzyme (ALAS).

There are two different forms of ALAS, the nonerythroid or hepatic form (ALAS1) and the erythroid form (ALAS2). Nonerythroid ALAS1 is coded for by a gene (chromosomal region 3p21) expressed in all tissues. The erythroid form, ALAS2, is coded for by a gene on the X chromosome (Xp21-q21). ALAS2 is the first and rate-limiting enzyme in heme synthesis.

Sex-linked SA (XLSA) is due to an abnormal *ALAS2* gene that is the result of heterogeneous mutations in the catalytic domain of the protein.[62] More than 22 different mutations have been described.[63] The mutations are located in exons 5–11 with most being single-base mutations affecting the site at which the enzyme binds the cofactor pyridoxal 5′-phosphate.

Evidence indicates that the activity of ALAS2 depends on the presence of pyridoxal phosphate. This cofactor binds to the enzyme and is crucial for its stability, maintenance of a conformation optimal for substrate binding and product release, and its catalytic activity. In 17 of the *ALAS2* mutations, a partial to complete clinical response to pharmacologic doses of pyridoxine occurs. Excess pyridoxine possibly enhances the abnormal ALAS enzyme activity by stabilizing it after synthesis.[62] In the pyridoxine-refractory sex-linked SA, the mutation appears to affect the processing of ALAS2 precursor mRNA (which terminates ALAS2 translation prematurely) or abolishes its enzymatic function.[63,64]

Significant ineffective erythropoiesis is characterized by bone marrow erythroid hyperplasia. This increased erythropoietic activity results in increased iron absorption in the gut. Iron overload can be significant and can lead to complications such as cardiac failure and diabetes.

Other hereditary forms of SA have been described but are less common than the sex-linked type.[65]

Acquired Sideroblastic Anemia

Acquired SA can be categorized as refractory anemia with ring sideroblasts (RARS) and those secondary to drugs or toxins. RARS is an acquired stem cell disorder and is discussed in the chapter on myelodysplastic syndromes (Chapter 25).

SA secondary to drugs or toxins is the result of the interference of the drugs or toxins with the activity of heme enzymes. Lead and alcohol are the most common causes of this form of SA.

Lead Poisoning. Lead poisoning **(plumbism)** has been recognized for centuries. In children, it generally results from ingestion of flaked lead-based paint. Lead was removed from paint sold after 1978. Children from lower socioeconomic backgrounds are at increased risk; those between 1 and 3 years of age are at greatest risk. Clinically, lead toxicity in children is associated with hyperactivity, low IQ, concentration disorders, hearing loss, and impaired growth and development. In adults, lead poisoning is primarily the result of inhalation of lead or lead compounds from industrial processes. It is the most common disease of toxic environmental origin in the United States.[66]

Many states require laboratories and physicians to report elevated blood lead levels to the state health department, which can report to the Centers for Disease Control and Prevention (CDC). Although average blood lead levels have dropped by 80% since the late 1970s, about 7,000 adults had blood lead levels of >25 mcg/dL in 2009 (40 states reporting).[67] In 2010, hundreds of thousands of children under age 6 were reported to have blood lead levels >5 mcg/dL, the level at which the CDC recommends public health actions be initiated (46 states reporting).[68] (In 2012, the CDC set a new acceptable blood lead level at for children [see Web Table 12-3]).

Lead serves no physiological purpose. Although lead poisoning consistently shortens the erythrocyte life span, the anemia accompanying plumbism is not primarily the result of hemolysis but of a marked abnormality in heme synthesis. Once ingested, lead passes through the blood to the bone marrow where it accumulates in the mitochondria of erythroblasts and inhibits cellular enzymes involved in heme synthesis. The heme enzymes most sensitive to lead inhibition are δ-aminolevulinic acid dehydrase (δ-ALA-D) and ferrocheletase (heme synthase) (Chapter 6). Other enzymes can be affected at higher lead concentrations. Thus, the synthesis of heme is disturbed at the conversion of δ-ALA to porphobilinogen (catalyzed by δ-ALA-D); urine excretion of δ-ALA increases as a result. Incorporation of iron into protoporphyrin to form heme (which uses the enzyme ferrochelatase) is also disrupted. The effect of lead on ferrochelatase is competitive inhibition with iron; iron accumulates in the cell, and EP (in the form of ZPP) is strikingly increased.

Studies have revealed that microcytic, hypochromic anemia is not characteristic of elevated lead levels in most children. Evidence suggests that the presence of a microcytic anemia in plumbism is most likely due to complications of ID or to the coexistence of

α-thalassemia trait.[69–71] One study found that 33% of African American children with lead poisoning and microcytosis had α-thalassemia trait.[69]

Coexistent ID and lead poisoning put children at a higher risk for developing even more serious complications because children absorb larger portions of lead in iron-deficient states and the competitive inhibition of ferrochelatase by lead is even greater in the absence of iron. Thus, it is critical to make a diagnosis of ID when it coexists with lead poisoning.

Although the ZPP measurement has been utilized to screen for lead in the past, it is no longer recommended as an appropriate screening tool because ZPP does not rise until lead levels reach approximately 20 mcg/dL.[72] Lead levels can impair neuropsychologic development. Screening generally should be done by direct lead measurements. ZPP also cannot be used to differentiate IDA and thalassemia in the presence of coexistent lead poisoning because of the increase in erythrocyte protoporphyrin caused by lead.

Alcoholism. Anemia is a common finding (up to 62%) in hospitalized chronic alcoholics.[31] Megaloblastic and sideroblastic anemia occur in the majority, but anemia in this population has many other causes including ACD, IDA, acute blood loss, and chronic hemolysis[73] (Table 12-12 ★). Less than one-half have an isolated cause for the anemia (Web Table 12-4). Studies show that sideroblastic anemia is particularly common among alcoholics with a poor diet and can be associated with a concomitant decrease in folic acid and megaloblastosis (Chapter 15). The presence of a dimorphic erythrocyte population and siderocytes in the peripheral blood are clues to a diagnosis of SA. Alcohol is believed to interfere with some enzymes of hemoglobin synthesis including inhibition of the synthesis of pyridoxal phosphate and of activity of uroporphyrinogen decarboxylase and ferrochelatase but enhancement of activity of δ-ALAS. Alcoholics with a poor diet can also have an inadequate intake of pyridoxine. Alcohol is directly toxic to hematologic cells as evidenced by the frequent presence of vacuoles in bone marrow precursor cells, thrombocytopenia, and granulocytopenia.

Interpreting laboratory tests in alcoholism is difficult because the alcohol has effects on multiple parameters. Even in the absence of megaloblastosis and anemia, the cells are frequently macrocytic (MCV 100–110 fL). Alcohol has a direct effect on folate metabolism, interfering with absorption, storage, and release. Serum iron levels are increased during drinking episodes but return to normal after cessation.

★ TABLE 12-12 Mechanisms of Anemia in Chronic Alcoholism

- Megaloblastosis associated with folate deficiency
- Iron-deficiency anemia from chronic or acute blood loss
- Sideroblastic anemia from toxic effects of alcohol on enzymes needed for heme synthesis
- Hemolytic anemia
  - Chronic hemolysis due to splenic sequestration
  - Spur cell anemia occurring in the setting of severe liver disease, splenomegaly, and jaundice
  - Transient hemolytic anemia associated with portal hypertension and acute congestive splenomegaly

Associated with Malignancy. Ring sideroblasts can be found in diseases other than sideroblastic anemia, including hematologic malignancies (e.g., leukemia, malignant histiocytosis, multiple myeloma, lymphoma) (Web Table 12-5). Some investigators believe that the presence of ring sideroblasts in these disorders suggests that the malignancy can result from an abnormal clone of pluripotential stem cells that affects the erythroid as well as other cell lineages. Occasionally ring sideroblasts appear in the bone marrow following treatment of malignant disease (e.g., multiple myeloma, Hodgkin's disease). The appearance of ring sideroblasts after treatment is considered a poor prognostic sign because these cases almost always terminate in acute leukemia.

Clinical Features

In patients with acquired sideroblastic anemias secondary to drugs or malignancy, the manifestations of the underlying disorder dominate. Patients with hereditary sideroblastic anemia or RARS, however, generally show primary signs and symptoms of anemia. In hereditary sideroblastic anemias, most patients also exhibit signs associated with iron overload including hepatomegaly, splenomegaly, and diabetes. In the latter stages of the disease, cardiac function can be affected.

Laboratory Features

Refer to Table 12-13 ★ for laboratory findings in sideroblastic anemia.

Peripheral Blood

The anemia is usually moderate to severe. A dimorphic picture of normochromic and hypochromic cells is characteristically seen in inherited and acquired secondary forms of SA (Figure 12-11 ■). Dual populations of macrocytes and microcytes or normocytes can be found. Hypochromic macrocytes are especially prevalent in RARS, whereas hypochromic microcytes are more common in the hereditary form of SA. Macrocytes are also common in SA associated with alcoholism.

If a dimorphic erythrocyte population is present, the MCV, MCH, and MCHC can be normal because these parameters represent an average of all erythrocytes, thus emphasizing the need for careful examination of CBC parameters and the blood smear. The RDW and erythrocyte histogram/cytogram are useful in detecting these dual populations. The RDW is increased, and the RBC histogram/cytogram shows two peaks representing the dual population.

★ TABLE 12-13 Laboratory Findings in Sideroblastic Anemia

- Dual population of hypochromic and normochromic erythrocytes
- Pappenheimer bodies in erythrocytes
- Normal or increased platelets
- Increased serum iron, serum ferritin, and percentage of saturation
- Ring sideroblasts in the bone marrow

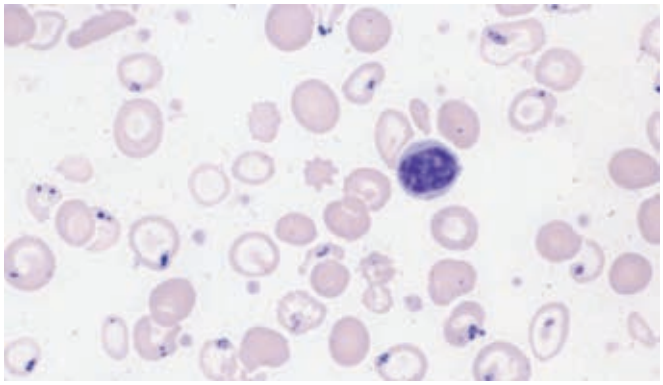


FIGURE 12-11 Blood film from a patient with sideroblastic anemia. Two populations of erythrocytes are present: hypochromic and normochromic. Anisocytosis is present with microcytes, macrocytes, and normocytes. This is the dimorphic blood picture typical of sideroblastic anemia. Note also the numerous inclusions (Pappenheimer bodies) (peripheral blood; Wright-Giemsa stain; 1000× magnification).

Other abnormalities of erythrocytes are often seen. Poikilocytosis and target cells can be present. Erythrocytes can contain **Pappenheimer bodies** (iron deposits) (Chapter 37). When Pappenheimer bodies are present, reticulocyte counts must be performed carefully because both RNA and Pappenheimer bodies take up supravital stains. However, Pappenheimer bodies stain with both Romanowsky and Prussian blue stains, whereas reticulated RNA does not stain with either. Nucleated erythrocytes are rarely present.

Basophilic stippling can be seen in any of the SAs. However, coarse punctate basophilic stippling, resulting from aggregated ribosomes and degenerating mitochrondria, is a particularly characteristic feature of lead poisoning (Figure 12-12 ■). The punctate stippling occurs in the reticulocyte and is found in developing erythroblasts. It has been shown that the granules in these stippled erythroblasts contain free ionized iron and the hemoglobin production in the cells is grossly deficient. Many stippled cells in the peripheral blood in lead poisoning are actually siderocytes.

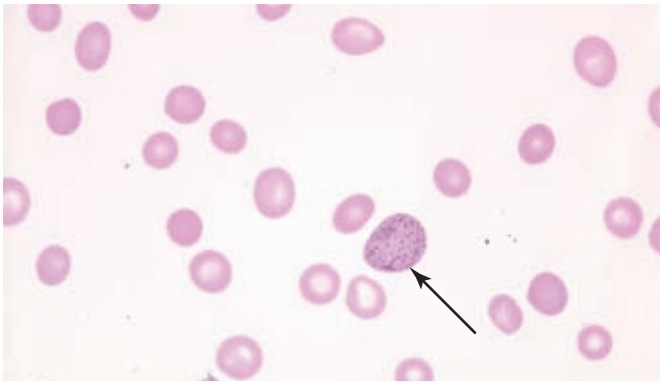


FIGURE 12-12 Peripheral blood smear from a patient with lead poisoning. Note the heavy basophilic stippling in the erythrocyte (peripheral blood; Wright-Giemsa stain; 1000× magnification).

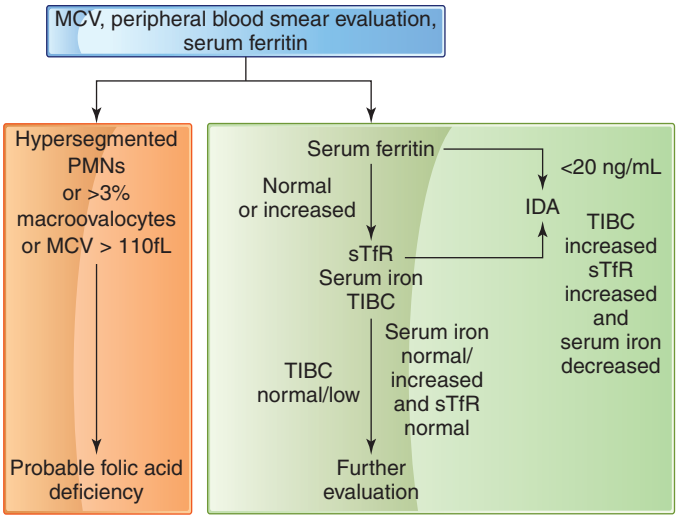


FIGURE 12-13 An algorithm for diagnosis of anemia in alcoholics.

Other laboratory test results can be abnormal. Even though the bone marrow is usually hyperplastic, the reticulocyte production index is <2, indicating that the anemia has an ineffective erythropoietic component. Other indications of ineffective erythropoiesis include a slightly increased serum bilirubin, (usually <2.0 mg/dL), decreased haptoglobin, and increased lactate dehydrogenase (LD). Iron studies show increased serum iron, normal or decreased TIBC with increased saturation levels (sometimes reaching 100%), and increased serum ferritin. Leukocyte and platelet counts are usually normal but can be decreased. Thrombocytosis is found in about one-third of patients.

In alcoholics, the direct and indirect effects of alcohol complicate the interpretation of laboratory tests used to diagnose anemia, including MCV, serum iron, TIBC, serum ferritin, and red cell and serum folate (Figure 12-13 ■).

CASE STUDY *(continued from page 215)*

8. Do the iron studies in Jose (serum iron 18 mcg/dL, TIBC 425 mcg/dL) suggest SA?

Bone Marrow

Bone marrow changes include erythroid hyperplasia often accompanied by various degrees of megaloblastosis. The megaloblastosis is sometimes responsive to folate, which indicates the presence of a complicating folate deficiency. Erythroblasts appear poorly hemoglobinized with scanty, irregular cytoplasm. Macrophages contain increased amounts of storage iron. Ring sideroblasts constitute more than 40% of the erythroblasts (Figure 12-14 ■). Ring sideroblasts are erythroblasts with iron granules that encircle one-third or more of the nucleus. The siderotic granules are larger than those found in normal sideroblasts. In hereditary SA, the abnormal granules occur primarily in the later stages of erythroblast development (e.g., polychromatophilic and orthochromatic erythroblast stages). In the idiopathic and secondary forms, the abnormal granules occur beginning with the earlier erythroblast stages.

Ring sideroblasts must be present for an SA diagnosis; however, it is important to recognize that other disease entities can have ring sideroblasts present without being SA.

Molecular Studies

If hereditary sex-linked SA is suspected based on erythrocyte morphology and iron studies, the patient should be tested for the presence of *ALAS2* gene mutations. This can be done by studying genomic DNA or cDNA (from RNA) from reticulocytes[63] (Chapter 42). If a mutation is found, other family members should be tested because even if anemia is not present, there is a risk of iron overload. Molecular studies also allow a distinction between hereditary SA and RARS.

CASE STUDY *(continued from page 219)*

9. Do Jose's laboratory test results and clinical history indicate that a bone marrow examination is necessary?

Therapy

Pyridoxine therapy is generally tried on patients with hereditary SA; <50% experience a return to normal hemoglobin levels with therapy, although some have a partial response. In some patients, iron overload decreases responsiveness to pyridoxine, but when the iron load is reduced by phlebotomy, hemoglobin concentration increases.[74] Folic acid is administered to those with megaloblastic features. Although the anemia can be treated, iron overload is the primary complication of SA. Sometimes the risk of hemochromatosis is lessened by the removal of excess body iron through phlebotomy or chelation therapy. Iron overload is monitored by iron studies (percent saturation of transferrin, serum ferritin). Female carriers should also be monitored for iron overload. Some patients live for many years tolerating their anemia well; others die because of complications of iron overload, infections, or bone marrow failure.

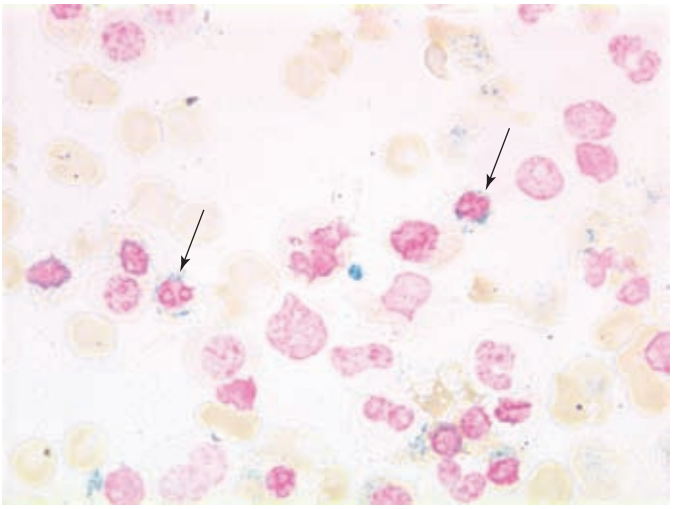


FIGURE 12-14 Ring sideroblasts (bone marrow; Prussian blue stain; 1000× magnification).

Secondary SA resulting from a disease, toxin, or drug may be corrected by successful treatment of the disease or by elimination of the toxin/drug.

HEMOCHROMATOSIS

Hemochromatosis describes the clinical disorder that results in parenchymal tissue damage from progressive iron overload. Hemochromatosis typically is not associated with anemia but is included in this chapter because iron studies are markedly abnormal in patients with this disorder. Hemochromatosis should be considered when symptoms are present, when there is a family history of the disorder, or when iron studies suggest iron overload. Iron overload is said to exist when serum ferritin is >200 mcg/L in premenopausal women or >300 mcg/L in men and postmenopausal women. A persistent transferrin saturation of ≥45% is often recommended as the value that indicates further investigation is necessary.[75] In hemochromatosis, excess iron deposits are stored not only in macrophages but also in hepatocytes, cardiac cells, endocrine cells, and other parenchymal tissue cells and interfere with the normal function of these cells. This situation has potentially fatal consequences, especially in relation to cardiac and hepatic dysfunction.

Hemochromatosis is classified as hereditary or secondary. The hereditary form is caused by genetic mutations. The secondary type is associated with other hematologic diseases and a variety of other conditions (Table 12-14 ★).

★ **TABLE 12-14** Causes of Iron Overload

Hereditary
• Classical hemochromatosis: *HFE*–associated mutations (type I)
• Juvenile hemochromatosis (type 2)
• Hemojuvelin (*HJV*) mutations (type 2A)
• Hepcidin (*HAMP*) mutations (type 2B)
• Transferrin-receptor 2 deficiency (type 3)
• Ferroportin deficiency (type 4)
• DMT1 mutations
• Congenital atransferrinemia
• Aceruloplasminemia
Secondary
• Anemias with ineffective erythropoiesis (e.g., sickle cell anemia, thalassemia, refractory anemias with erythroid hypercellular bone marrow, X-linked sideroblastic anemia)
• Chronic transfusions
• Chronic liver disease
• Viral hepatitis
• Dietary iron overload
• Overload from injections or ingestion of iron supplements
• Alcoholism
• Insulin-resistance-associated hepatic iron overload
• African dietary iron overload
• Porphyria cutanea tarda
• Medicinal iron ingestion

Hereditary Hemochromatosis

Etiology and Pathophysiology

Hereditary hemochromatosis (HH) is characterized by increased iron absorption in the gut and progressive iron overload. It is a genetic disorder with a prevalence of 1 in 200–250 persons. One in ten Caucasians in the United States is a carrier. It is the most common genetic abnormality in those with a European ancestry. Most of the hereditary forms of iron overload involve a deficiency of hepcidin either as a result of a mutation involving the hepcidin gene (*HAMP*) or mutations of genes whose products regulate hepcidin expression (HFE, TfR2, HJV, DMT1, transferrin). The result is an increase in absorption of iron in the intestine and/or uncontrolled release of iron from macrophages and duodenal enterocytes into the plasma iron pool. Iron in excess of that needed for erythropoiesis and myoglobin production is deposited in hepatocytes and parenchymal tissue cells, resulting in an increase in total body iron stores. Most mutations in genes that affect the regulation of iron metabolism are recessive disorders (Table 12-15 ★).

The most common form of HH in populations of European origin is due to mutations in the *HFE* gene, identified in 1996.[76] Mutations result in an autosomal recessive disorder of low penetrance with adult onset. Clinical disease is more common in males than females. The *HFE* gene is mapped to chromosome 6p21,3. The most common mutation is cys282 ⟶ tyrosine: C282Y. Less common are the mutations his63 ⟶ asp: H63D; and ser65 ⟶ cys: S65C. Clinical signs of the disease can be found in homozygous C282Y and compound heterozygosity for C282Y and H63D. The mechanism of the way mutations in the HFE protein affect iron metabolism is unclear but can involve interactions of HFE with other proteins in addition to TfR (see earlier discussion of "Systemic Iron Balance, HFE"). Finding decreased hepatic expression of hepcidin in *HFE*-related HH suggests that the mutation affects upstream regulation of hepcidin. About 3–4 mg of iron a day is absorbed from the GI tract as compared with the usual 1–2 mg, resulting in an accumulation of about 0.5–1.0 gm/year. The capacity of cells to store iron by complexing with apoferritin is exceeded, and "free" intracellular iron accumulates. This free iron facilitates the buildup of reactive oxygen species that cause cell injury and cell death and leads to organ failure. Most of the excess iron is deposited in the liver. Mutations of the *TfR2* gene result in a disease that is clinically indistinguishable from *HFE*-associated HH.

Other less common forms of HH are due to mutations in other genes involved in iron sensing and regulation, but all appear to result in deregulation of hepcidin expression.

Mutations in the ferroportin gene can decrease the amount of functional ferroportin on the cell surface, blocking iron export and resulting in retention and accumulation of iron in macrophages. This

★ **TABLE 12-15** Summary of Acquired and Hereditary Iron Overload Disorders

Disorder	Defect/Mutation	Fe Absorption	Fe Deposition Site(s)	Plasma Iron	Ferritin
Acquired					
Iron-loading anemias (ineffective erythropoiesis; e.g., thalassemia, XLSA)	Erythropoietic drive and possibly GDF-15 suppress hepcidin synthesis	Increased	Macrophage	Increased	Increased
Anemia of chronic disease (ACD)	IL-6 increases hepcidin, which binds ferroportin and traps iron in macrophage	Decreased	Macrophage	Decreased	Normal to Increased
Transfusion induced	Gain of 1 mg iron/mL erythrocytes transfused	Normal	Parenchymal tissue	Increased	Increased
Hereditary					
Hereditary hemochromatosis (autosomal recessive)	*HFE* gene, 6p21; *TfR2* gene, 7q22	Increased	Parenchymal tissue	Increased	Increased
Juvenile hemochromatosis (autosomal recessive)	*HJV* gene 1q21; *HAMP* (hepcidin) gene, 19q13; *HFE* and *TfR2* (TfR2) genes	Increased	Parenchymal tissue	Increased	Increased
Hereditary hemochromatosis (autosomal dominant)					
(a) Ferroportin associated with impaired iron export	*SLC40A1* (Ferroportin) gene, 2q32	Normal or low but defective export of iron from macrophages	Predominantly deposition in macrophages	Normal but increases with progression of disease	Increased
(b) Ferroportin associated with hepcidin resistance	*SLC40A1* gene, 2q32	Normal but increased export of iron from duodenal enterocytes	Parenchymal tissue: heart, liver, pancreas, other organs	Increased	Increased

type of mutation produces only minor clinical manifestations. Alternatively, mutations can cause resistance of ferroportin to internalization and degradation induced by hepcidin. The result is loss of control of iron export from enterocytes and macrophages, which leads to iron overload in parenchymal cells of the liver and other organs. Ferroportin mutations are inherited as dominant disorders. Aceruloplasminemia affects expression of ferroportin on the cell surface leading to ferroportin degradation.

Clinical Findings

Clinical penetrance of the most common form of HH, *HFE*-mutation, is incomplete with only 1 in 5,000 affected individuals having clinical symptoms. About 81% of symptomatic patients have the C282Y/C282Y genotype. Asymptomatic patients who have the C282Y/C282Y genotype or the H63D/C282Y genotype should have their serum iron and ferritin levels monitored because they are at risk for developing iron storage disease. Clinical findings include chronic fatigue, arthralgia, infertility, impotence, cardiac disease, diabetes, and/or cirrhosis.[77] Hyperpigmentation of the skin is also found.

Laboratory Findings

Screening tests for hemochromatosis usually include percent saturation of transferrin and serum ferritin. The criterion for a diagnosis of hemochromatosis is usually ≥45% transferrin saturation. Saturation often approaches 100%. Adding serum ferritin analysis increases specificity for HH; iron overload is suggested when the serum ferritin is elevated. Although an increase in transferrin saturation is sensitive for hemochromatosis, DNA testing to identify the mutated gene is necessary for a definitive diagnosis. Biopsy of the liver and/or bone marrow usually is not needed. Liver enzymes are often elevated. Anemia is not characteristic except in aceruloplasminemia (when a normocytic, normochromic anemia is present) and in *DMT1* mutations (when a severe microcytic, hypochromic anemia is present).[78]

Screening for HH is controversial. Some recommend population screening with transferrin saturation testing and suggest that laboratories include transferrin saturation in their routine lab panels.[79] Genetic screening is still costly for population screening, and unresolved ethical issues including patient privacy, counseling, and insurance concerns exist. With the recognition of the low penetrance of clinical disease (<1%), interest in large-scale screening has largely disappeared. It is recommended, however, that both symptomatic and asymptomatic first-degree relatives of those with HH be tested with genetic screening because transferrin saturation will not detect carriers.[80] The goal of screening and identifying the disease early is to allow treatment with phlebotomy before the onset of clinical disease.

Secondary Hemochromatosis

Secondary hemochromatosis is associated with a number of conditions including anemias that have an ineffective erythropoiesis component and increased iron absorption. These include β-thalassemia, congenital dyserythropoietic anemia, and X-linked sideroblastic anemia. An increase in GDF-15 associated with ineffective erythropoiesis could be responsible for suppressing hepcidin synthesis, leading to an increase in iron absorption in these conditions. Iron overload often develops in patients who have transfusion-dependent anemias such as sickle cell disease and thalassemia. Iron overload can also be found in chronic liver disease. Serum iron studies are abnormal in 40–50% of patients with chronic viral hepatitis, alcoholic liver disease, and nonalcoholic steatohepatitis. Alcohol disrupts normal iron metabolism and results in excess iron deposition in the liver in about one-third of alcoholics.[81]

Treatment

Phlebotomy is usually the treatment for HH. Each unit of blood removes about 250 mg of iron from the body. It is suggested that following initial venesection therapy to deplete iron stores, iron status be monitored annually by serum ferritin levels.[82]

Treatment of secondary hemochromatosis sometimes includes iron chelation (e.g., deferoxamine) to bind iron and enable urinary excretion. Only a very small amount of iron is available for chelation (from the labile pool) at any given time; thus, continuous infusion of the chelator over a long period of time is required to remove this iron. Chelation therapy is the primary form of treatment for transfusion-induced hemochromatosis.

☑ CHECKPOINT 12-5

What is the risk of population genetic screening for HH? What is the benefit of population genetic screening for HH?

PORPHYRIAS

Heme is an iron-chelated porphyrin ring. Its biosynthesis occurs in most cells, but the major sites of synthesis are erythroid cells of the bone marrow and hepatocytes. Thus, the activity of the enzymes catalyzing porphyrin metabolism is highest in these cells.

The porphyrias represent a group of inherited disorders characterized by a block in porphyrin synthesis. Abnormal porphyrin metabolism is due to a defect in one or more of the enzymes in the heme synthesis pathway. As a result of these enzyme deficiencies, the porphyrin precursors behind the enzyme defect accumulate in tissues, and large amounts are excreted in the urine and/or feces. These excess porphyrin deposits cause most of the symptoms and clinical findings associated with porphyria. The most common findings include photosensitivity, abdominal pain, and neuropathy (motor dysfunction, sensory loss, mental disturbances). However, usually adequate production of heme for hemoglobin synthesis occurs.

Although rare, the porphyrias have received wide recognition and stimulated interest because the disease affected the royal families of England and Scotland, especially those descended from Mary, Queen of Scots. Historians have described George III as suffering from a mental and physical disorder thought to have been porphyria. Princess Charlotte, the granddaughter of George III and heir to the throne, died in childbirth; her death was attributed to an acute porphyria attack at the time of delivery.

Two forms of porphyria (erythropoietic and hepatic) are described, depending on the primary site (bone marrow or liver) of defective porphyrin metabolism (Table 12-16 ★). Only the erythropoietic porphyrias affect the erythrocytes. Therefore, these porphyrias are discussed here.

★ **TABLE 12-16** Classification and Characteristics of the Porphyrias

Porphyria	Mode of Inheritance	Enzymatic Defect	Metabolites in Excess			
			Urine	Feces	Erythroid Cells	Tissue Source
Erythropoietic						
Congenital erythropoietic porphyria (CEP)	Autosomal recessive	Uroporphyrinogen III synthase	Uroporphyrin I, Coproporphyrin I	Coproporphyrin I, Uroporphyrin I	Uroporphyrin I, Coproporphyrin I	Erythropoietic
Erythropoietic protoporphyria (EPP)	Autosomal dominant	Ferrocheletase (heme synthase)	Normal	Protoporphyrin	Protoporphyrin	Erythropoietic and occasionally hepatic
Hepatic and Erythropoietic						
Hepatoerythropoietic porphyria (HEP)	Recessive	Uroporphyrinogen decarboxylase	Uroporphyrin	Uroporphyrin	ZPP	Hepatic and erythropoietic
Hepatic						
Acute intermittent porphyria (AIP)	Autosomal dominant	Porphobilinogen deaminase	δ-ALA, Porphobilinogen	Sometimes Coproporphyrin and Protoporphyrin	Normal	Hepatic
Hereditary coproporphyria (HCP)	Autosomal dominant	Coproporphyrinogen oxidase	Coproporphyrin III, Uroporphyrin (in attack)	Coproporphyrin III	Normal	Hepatic
Variegate porphyria (PV)	Autosomal dominant	Protoporphyrinogen oxidase	Porphobilinogen, δ-ALA, Uroporphyrin, Coproporphyrin	Protoporphyrin Coproporphyrin	Normal	Hepatic
Porphyria cutanea tarda (PCT)	Autosomal dominant	Uroporphyrinogen decarboxylase	Uroporphyrin I, Uroporphyrin III, Coproporphyrin (slight)	Protoporphyrin Coproporphyrin	Normal	Hepatic
ALA dehydratase deficiency porphyria (ADP)	Autosomal recessive	ALA dehydratase	δ-ALA, Coproporphyrin III	—	Normal	Hepatic

Pathophysiology

The erythropoietic porphyrias result from an abnormality of the enzymes in the heme biosynthetic pathway within the erythroblasts of the bone marrow. At least two types exist: congenital erythropoietic porphyria (CEP) and erythropoietic protoporphyria (EPP). They are classified according to the particular enzyme defect and the excessive porphyrin intermediates produced. Although CEP is associated with hemolytic anemia, anemia in EPP is rare.

Porphyrins are functionless products produced by the irreversible oxidation of type I and type III porphyrinogens. Porphyrinogens of the type III series are the precursors of heme, whereas the type I isomers do not produce any useful metabolites and cannot be used in heme synthesis (Chapter 6). In normal heme synthesis, both type I and III isomers are formed in a 1:10,000 ratio. Normally, most porphyrinogens are readily converted to heme, and very small amounts of the porphyrin intermediates are formed. If for some reason excessive amounts of porphyrinogens are produced, the corresponding oxidized porphyrin compounds also increase. Porphyrins are tetrapyrroles and resonating compounds (contain alternating single and double bonds); erythrocytes that contain these substances show red fluorescence with UV light.

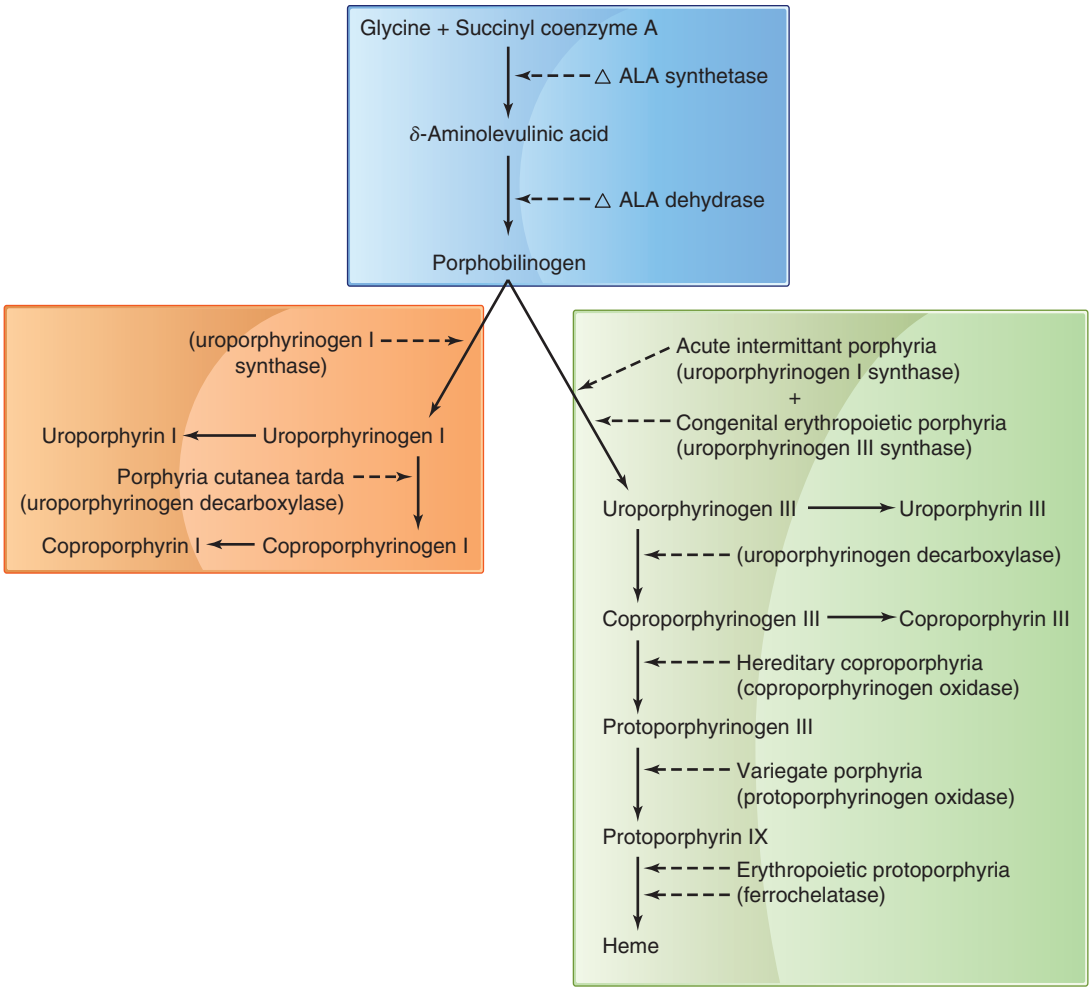
Congenital Erythropoietic Porphyria (CEP)

CEP (Gunther's disease) is characterized by the presence of excessive amounts of type I porphyrins: uroporphyrin I (UroI) and coproporphyrin I (CoproI). A defect in uroporphyrinogen III synthase results from point mutations in the gene, shifting the porphobilinogen into the functionless UroI isomer (Figure 12-15 ■). However, enough UroIII is produced to generate adequate amounts of heme. This suggests that a deficiency of uroporphyrinogen III synthetase is not the only abnormality. Another possibility for the excessive amounts of UroI isomer is hyperactivity of uroporphyrinogen I synthase. Validation of either of these hypotheses requires purification, characterization, and accurate measurement of these enzymes.

The excess porphyrins are deposited in body tissues and excreted in urine and feces. Intense fluorescence with ultraviolet light can verify their presence. The cause of the hemolytic anemia that accompanies CEP is unclear but is thought to be associated with the excessive porphyrin deposits within erythrocytes. The finding that normal erythrocytes infused into CEP patients have a normal life span supports this. The erythrocytes in CEP can be subject to photohemolysis as they pass through the dermal capillaries exposed to UV light. The erythrocytes show increased photohemolysis in vitro, but whether this occurs in vivo is uncertain.

Erythropoietic Protoporphyria (EPP)

EPP is characterized by an overproduction of protoporphyrin, the immediate precursor of heme, and is associated with a defect in ferrochelatase (Figure 12-10). Adequate amounts of heme are produced, however, and no anemia is present. Excess protoporphyrin IX builds



■ **FIGURE 12-15** The formation of heme from glycine and succinyl-coenzyme A involves the production of porphyrinogen intermediates. Normally, only very small amounts of the series I isomers are formed. However, in a group of congenital disorders called porphyrias, there is a block in porphyrin metabolism due to a defect in one or more of the enzymes involved in porphyrin metabolism, and there is a build-up of porphyrin intermediates depending on which enzyme is involved. In congenital erythropoietic porphyria, there is an abnormality in conversion of porphobilinogen to uroporphyrinogen III, and large amounts of the functionless series I isomers are formed. These isomers are oxidized to porphyrin and accumulate in the tissues. Another form of porphyria, erythropoietic protoporphyria, is characterized by excessive production of protoporphyrin due to an abnormality of the ferrochelatase enzyme. Other porphyrias noted in this figure are classified as hepatic because the site of abnormal metabolism is the liver.

up in the cell, can leak into skin dermal capillaries, and can be found in the skin, liver, blood, and feces. Due to its insolubility in water, protoporphyrin is not present in the urine. This porphyria is thought to be of both erythropoietic and hepatic origin.[83]

Clinical Features

CEP is a rare autosomal recessive disease with ~130 cases reported. EPP is inherited as an autosomal dominant trait, but recessive inheritance is possible.[84] About 300 cases of EPP have been reported, but the actual rate of occurrence is probably masked due to the subtlety of the clinical signs and the absence of colored porphyrins in the urine.

The first signs of CEP occur in infancy. The urine is colored pink to reddish brown, depending on the amount of uroporphyrin excreted. This is usually first noted as a pink stain on the infant's diaper. The excess porphyrins in the skin create an extreme photosensitivity to sunlight. Vesicular or bullous eruptions appear on uncovered areas shortly after exposure to sunlight. The lesions heal slowly and can become infected. Repeated eruptions and skin injury cause scarring and can lead to severe mutilation of the face, ears, and hands. The excess porphyrin stains the teeth a brown discoloration. Under UV light, the teeth fluoresce bright red. Hypertrichosis affects the entire body but is especially present in exposed areas. The hair can be blond and downy or dark and coarse. Splenomegaly is a consistent finding

and is usually progressive with the disease. A mild to severe hemolytic anemia is present with erythrocyte life span decreased to as little as 18 days. Patients with CEP do not exhibit the abdominal pain or neurologic and psychotic signs associated with some hepatic porphyrias.

Clinical signs of EPP are more subtle, and its course is relatively mild in comparison with CEP. Photosensitivity is not severe, and scarring is usually absent. Sunlight exposure leads to erythema and urticaria. Protoporphyrin accumulates in the erythrocytes, which causes them to fluoresce intensely, but there is no hemolytic anemia. Occasionally hepatic damage occurs.

Laboratory Features

Congenital Erythropoietic Porphyria

The peripheral blood in CEP exhibits a mild to severe normocytic anemia with anisocytosis and poikilocytosis. The blood smear reveals significant polychromatophilia and nucleated erythrocytes. The erythrocytes fluoresce with UV light.

The bone marrow in CEP shows erythroid hyperplasia. A large portion of the erythroblasts demonstrates intense fluorescence with UV light. The fluorescence is localized principally in the nuclei. The fact that not all erythroblasts fluoresce suggests that two populations of erythrocytes exist, one of which is normal.

Serum iron and storage iron are usually normal. Haptoglobin is absent, and unconjugated bilirubin as well as urinary and fecal urobilinogen are increased.

Large amounts of uroporphyrin I and coproporphyrin I are excreted in the urine and feces. These isomers are also found in the plasma and in erythrocytes.

Erythropoietic Protoporphyria (EPP)

The blood and bone marrow in EPP usually reveal no abnormalities on routine examination; however, under UV light, the cytoplasm of erythroblasts fluoresces intensely. The erythrocytes, plasma, and feces contain large amounts of protoporphyrin; protoporphyrin is not found in the urine. The protoporphyrin in erythrocytes is free (FEP), not bound to zinc as in IDA and lead poisoning. The FEP is higher than in other disorders associated with an increase in erythrocyte protoporphyrin levels. This block in heme synthesis, which occurs in the reaction just prior to insertion of iron into the porphyrin ring, would be expected to cause an accumulation of iron within the erythroblasts. The fact that this iron buildup does not occur has not been explained.

Prognosis and Therapy

Individuals with CEP do not usually survive beyond the fifth decade of life. Attempts to decrease the excess porphyrins have been unsuccessful, but the quality of life for CEP patients has improved by minimizing the scarring and mutilation with effective dermatologic treatment. Avoiding exposure to sunlight is critical. Splenectomy has sometimes resulted in a decrease of porphyrin production and helped ameliorate the hemolytic anemia. Evidence for long-term success with splenectomy, however, is questionable. Blood transfusion in conjunction with administration of chelators to reduce iron overload suppresses erythropoiesis and decreases or eliminates symptoms. Bone marrow transplantation is suggested in severe phenotypes because the predominant site of porphyrin production is the bone marrow.

Treatment of EPP is to protect the skin from sunlight and minimize the toxic effects of protoporphyrin on the liver. In most patients with EPP, high doses of δ-carotene improve tolerance to sunlight. Blood transfusions and hematin can be utilized to suppress erythropoiesis. Splenectomy can be helpful if hemolysis and splenomegaly are prominent. Cholestyramine can promote excretion of liver protoporphyrin.[85]

Genes of the heme biosynthetic pathway have been cloned, and mutations associated with porphyrias have been identified. Gene therapy may be an option for porphyrias in the future.[82] This type of therapy involves inserting a functional gene into specific hematopoietic or hepatic stem cells of the patient, restoring normal heme synthesis pathways.

Summary

Hemoglobin synthesis requires adequate production of heme and globin. Inadequate amounts of either can result in anemia—usually microcytic, hypochromic anemia. Defects in heme synthesis could be due to either faulty iron or porphyrin metabolism (Table 12-17 ★).

Many proteins, including hepcidin, ferroportin1, hephaestin, DMT1, DCytB, HFE, TfR, GDF-15, HIF-2, and transferrin, play a role in iron homeostasis. The anemias with a faulty iron metabolic component include IDA and ACD. IDA is due to inadequate amounts of iron for heme synthesis; ID usually occurs because of blood loss or a nutritional deficiency of iron. ACD has several pathophysiologic mechanisms related to cytokines produced as a result of inflammation or infection. The major mechanism is a block in reutilization of macrophage iron. The erythrocytes are normocytic, normochromic, but in long-standing anemia, they can be microcytic, hypochromic.

Abnormal porphyrin metabolism results in SA and porphyrias. SA is due to defective porphyrin synthesis and a block in the insertion of iron into the porphyrin ring to form heme. The erythrocyte population in SA characteristically contains cells that are normochromic and hypochromic (dual population). The bone marrow has ring sideroblasts. Hereditary SA is due to defective ALAS2, the enzyme in the first step of heme synthesis. SA can also occur because of the effects of drugs or toxins on enzymes involved in heme synthesis.

Iron studies are helpful in differentiating these disorders. Serum iron is decreased in IDA and ACD; it is normal to increased in SA. The serum transferrin is increased and saturation decreased if total body iron is decreased, whereas if storage iron is normal or increased, the serum transferrin is normal or decreased with normal or increased saturation. Serum ferritin is a reliable indicator of iron stores except in the presence of inflammation or infection

when it can be falsely increased. Serum ferritin is decreased in ID and normal or increased in SA, ACD, and hemochromatosis. The serum transferrin receptor assay is useful in differentiating ID and ACD. Levels are increased in ID and normal in ACD.

Hemochromatosis is a disorder characterized by total body iron excess. Anemia is not a characteristic of this disorder, but it is included in this chapter to help the reader compare and differentiate iron study results with those associated with defective heme synthesis. Hemochromatosis can be caused by a genetic defect or chronic transfusions. The most common hereditary form of hemochromatosis (HH) is due to a mutation of the *HFE* gene. Mutations in several other genes associated with iron metabolism can also cause HH. These mutations are believed to affect the synthesis or function of hepcidin. Serum iron studies reflect the excessive iron overload with increased serum ferritin and a very high saturation of transferrin.

Porphyria is a heterogeneous group of hereditary disorders due to a block in porphyrin synthesis. The defect involves one of the critical enzymes in the porphyrin metabolic pathway. Two forms, CEP and EPP, have an erythropoietic component. Erythrocytes have very high levels of free erythrocyte protoporphyrin, and excess porphyrins are deposited in tissues and excreted in feces/urine.

★ **TABLE 12-17** Summary of Conditions Associated with Abnormal Heme Synthesis and/or Iron Metabolism/Utilization

Condition	Etiology	CBC	Iron Studies	Other
Iron-deficiency anemia	Inadequate iron due to deficient dietary intake; decreased absorption; increased loss	Microcytic, hypochromic, anisocytosis, poikilocytosis	Decreased serum iron, serum ferritin, and percent saturation of transferrin; increased TIBC and sTfR	Increased ZPP (do not use to evaluate if possibility of concurrent lead poisoning); decreased reticulocyte hemoglobin (CHr, MCHr, RET-He); increased sTfR-F index
Anemia of chronic disease	Impaired release of iron from macrophages due to increased hepcidin induced by cytokines; inhibition of EPO production and impaired erythropoiesis; shortened erythrocyte survival	Normocytic, normochromic; in long-standing cases can be microcytic, hypochromic	Decreased serum iron and low to normal TIBC; low/normal transferrin saturation; increased or normal serum ferritin; sTfR normal	Increased ZPP, decreased sTfR-F index
Sideroblastic anemia	Defect in enzymes needed for heme synthesis; can be hereditary or acquired (secondary to drugs/toxins)	Dual population of normochromic and hypochromic erythrocytes and macrocytic, microcytic or macrocytic, normocytic erythrocytes	Increased serum iron and transferrin saturation; normal or decreased TIBC; increased serum ferritin	Bone marrow shows ring sideroblasts; peripheral blood erythrocytes can contain Pappenheimer bodies
Hemochromatosis (no anemia)	Hereditary: genetic mutations resulting in deficiency of ferroportin or hepcidin; acquired: transfusion, iron injections, chronic liver disease	No anemia or characteristic peripheral blood cell morphology except in DMT1 and ferroportin −1 mutations	Increased serum iron; transferrin saturation (≥45%), and increased serum ferritin	Molecular testing to identify mutated gene is suggested if transferrin saturation is more than 45%
Porphyrias (anemia not characteristic)	Block in porphyrin synthesis due to defect in enzymes for heme synthesis; CEP and EPP affect erythrocytes	CEP: normocytic hemolytic anemia; anisocytosis and poikilocytosis; polychromatophilia; nucleated RBCS EPP: no abnormalities	Storage iron and serum iron normal	CEP: increased uroporphyrin I and coproporphyrin I excreted in urine and feces, plasma, and erythrocytes; series III isomers also increased CEP, EPP: normoblasts demonstrate intense fluorescence with UV light

Review Questions

Level I

1. What is the iron transport protein called? (Objective 3)
   A. ferritin
   B. transferrin
   C. hemosiderin
   D. albumin
2. The term *sideropenic* is most closely associated with which of the following anemias? (Objective 1)
   A. iron deficiency
   B. sideroblastic
   C. lead poisoning
   D. anemia of chronic disease
3. Microcytic, hypochromic erythrocytes are most characteristic of which of the following anemias? (Objective 5)
   A. megaloblastic
   B. lead poisoning
   C. iron deficiency
   D. anemia of chronic disease
4. Which of the following individuals is most likely to require an increased intake of iron? (Objective 4)
   A. adult male
   B. menopausal female
   C. mother of three preschool children
   D. 75-year-old male
5. The basic defect in sideroblastic anemia is: (Objective 6)
   A. inadequate iron intake
   B. inadequate absorption of iron in the gut
   C. cytokine inhibition of erythropoiesis
   D. defect in enzymes regulating heme synthesis
6. Anemia(s) characterized by defective heme synthesis includes: (Objective 6)
   A. hemochromatosis
   B. megaloblastic anemia
   C. thalassemia
   D. sideroblastic anemia
7. The most common cause of ID in middle-aged men is: (Objective 6)
   A. inadequate iron in the diet
   B. cancer
   C. prescription drugs
   D. chronic bleeding
8. Which of the following is most often associated with microcytic, hypochromic anemia? (Objective 5)
   A. lead poisoning
   B. ID
   C. sideroblastic anemia
   D. anemia of chronic disease
9. Which of the following best describes hemosiderosis? (Objective 7)
   A. decrease in serum iron
   B. increase in serum iron
   C. increase in macrophage iron
   D. increase in total body iron
10. A patient with anemia of chronic disease would be expected to have which set of laboratory test results? (Objective 5)
    A. MCV decreased, serum iron increased, serum ferritin increased, TIBC and percent saturation increased
    B. MCV normal, serum iron increased, serum ferritin decreased, TIBC and percent saturation decreased
    C. MCV normal, serum iron decreased, serum ferritin increased, TIBC and percent saturation decreased
    D. MCV decreased, serum iron decreased, serum ferritin decreased, TIBC and percent saturation decreased

Level II

Use this history for questions 1–4.

A 75-year-old male experiencing mental confusion and fatigue was seen by his physician. Laboratory tests were ordered:

RBC	3.3×10^{12}/L
Hb	9.3 g/dL
Hct	0.29 L/L
PLT	168×10^{9}/L
WBC	4.0×10^{9}/L
Differential	
Segmented neutrophils	60%
Band neutrophils	9%
Lymphocytes	25%
Monocytes	3%
Eosinophils	3%

RBC morphology: Anisocytosis with microcytic, hypochromic RBCs, and normocytic, normochromic RBCs present
Laboratory data for anemia workup:

Reticulocyte count	1.0%
Serum iron	274 mcg/dL
Total iron-binding capacity (TIBC)	285 mcg/dL

1. Which anemia of defective heme synthesis is associated with this type of red cell morphology? (Objective 8)

   A. sideroblastic anemia

   B. anemia of chronic disease

   C. iron-deficiency anemia

   D. erythropoietic porphyria

2. Which laboratory result(s) is (are) most useful in distinguishing this patient's anemia from IDA? (Objective 9)

   A. bone marrow

   B. mean cell volume

   C. hemoglobin

   D. iron studies

3. From the results of these laboratory studies, how would you describe the patient's red blood cells? (Objective 8)

   A. microcytic, hypochromic

   B. dual population of microcytes and normocytes

   C. macrocytic, normochromic

   D. normocytic, normochromic

4. A bone marrow examination was performed and sections were stained with Prussian blue. Numerous sideroblasts were present with a large number of ring sideroblasts. What is the most probable cause of this anemia? (Objectives 6, 8, 10)

   A. poor diet

   B. chronic blood loss

   C. abnormality of ALAS2

   D. increased iron requirement

5. A hematocrit is not recommended to screen for iron deficiency in children because: (Objectives 1, 2, 8, 9)

   A. it is not sensitive enough to pick up anemia in children

   B. iron deficiency can be present without the presence of anemia

   C. high levels of lead will affect the hematocrit accuracy

   D. serum ferritin is more cost effective

6. If a child with lead poisoning also had a significant microcytic, hypochromic anemia, what complicating pathology/pathologies should be considered? (Objective 14)

   A. iron deficiency

   B. thalassemia

   C. iron deficiency and thalassemia

   D. thalassemia and sideroblastic anemia

7. What laboratory test is best for screening for iron deficiency in a population of 1- to 3-year-old children who have a high incidence of elevated blood lead levels? (Objective 14)

   A. hematocrit

   B. serum ferritin

   C. serum iron

   D. ZPP

8. A 2-year-old child was tested for blood lead level. The result was 25 mcg/dL. He also had microcytic, hypochromic anemia. The child's parents were questioned, and it was determined that the source of lead was a painted crib in his day care center. The child was enrolled in another day care center. Follow-up testing revealed that the blood lead level was within normal limits but the microcytic, hypochromic anemia was still present. Which follow-up test(s) would you recommend to help identify the etiology of this anemia? (Objectives 9,14,15)

   A. serum iron, serum ferritin, TIBC, % saturation

   B. hemoglobin electrophoresis

   C. molecular diagnostic testing for sideroblastic anemia

   D. molecular diagnostic testing for hemosiderosis

9. A health maintenance organization (HMO) has a contract for laboratory testing services with your laboratory. The HMO has decided to screen its members for hereditary hemochromatosis. Which laboratory test will you recommend for this screening? (Objective 18)

   A. serum iron

   B. % transferrin saturation

   C. serum ferritin

   D. molecular test for mutated *HFE* gene

10. In regard to question 9, what test should you recommend be done reflexively on patients with an abnormal screening test? (Objective 18)

    A. molecular test for *HFE* gene

    B. serum iron

    C. serum ferritin

    D. % saturation

11. Which protein regulates iron absorption in the gut and transport of iron from cells to the plasma? (Objective 3)
    A. DcytB
    B. DMT1
    C. hepcidin
    D. hephaestin

12. What effect would a high degree of ineffective erythropoiesis have on iron metabolism? (Objective 4)
    A. decreased absorption of iron in the intestine
    B. decreased transport of iron across the basolateral membrane of enterocytes
    C. decreased hepcidin synthesis
    D. increased serum transferrin saturation

Companion Resources

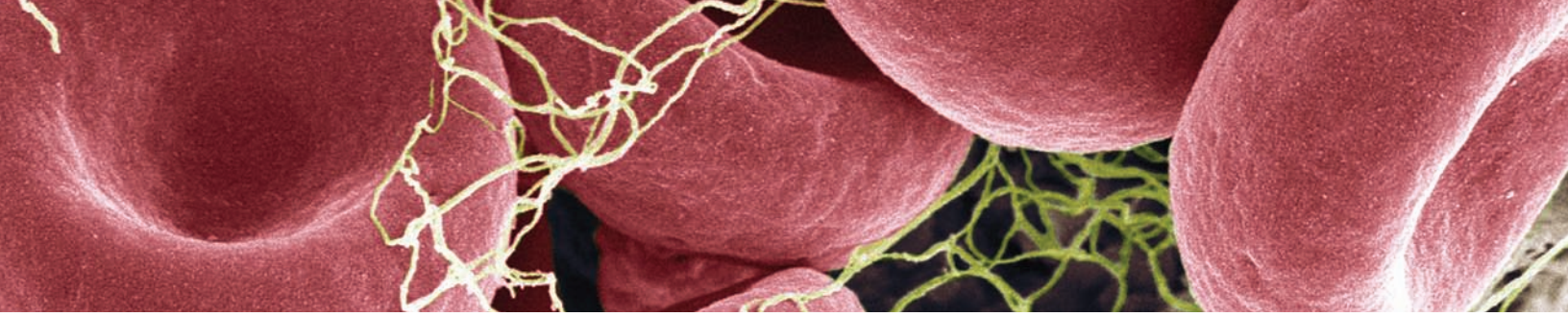
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Andrews NC. Pathology of iron metabolism. In: Hoffman R, Benz Jr. EJ, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*. 5th Ed. New York: Churchill Livingstone; 2009 (available at www.mdconsult.com/books/page.do?eid=44-u1.0-B978-0-443-06715-0..X5001-8&isbn=978-0-443-06715-0&uniqId=350057742-2\#4-u1.0-B978-0-443-06715-0..X5001-8--TOP; accessed August 6, 2012).
2. Roy CN, Andrews NC. Anemia of inflammation: the hepcidin link. *Cur Opi Hemat*. 2005;12(2):107–11.
3. Guschin H, Mackenzie B, Berger UV et al. Cloning and characterization of a mammalian proton-coupled metal-iron transporter. *Nature*. 1997;388:482–88.
4. Qiu A, Jansen M, Sakaris A et al. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell*. 2006;127:917–28.
5. Anderson GJ, Frazer DM, McLaren GD. Iron absorption and metabolism. *Curr Opin Gastroenterol*. 2009;23:129–35.
6. Gavin MW, McCarthy DM, Garry PJ. Evidence that iron stores regulate iron absorption: a setpoint theory. *Am J Clin Nutr* 1994;59:1376–80.
7. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. *Cell*. 2004;117:285–97.
8. Frazer DM, Wilkins SJ, Becker EM et al. A rapid decrease in the expression of DMT1 and DCytb but not Ireg1 or hephaestin explains the mucosal block phenomenon of iron absorption. *Gut*. 2003;52(3):340–46.
9. Nicolas G, Chauvet C, Viatte L et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anaemia, hypoxia, and inflammation. *J Clin Invest*. 2002;110:1037–44.
10. Tanno T, Bhanu NV, Oneal PA et al. High levels of GDF-15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nat Med*. 2007;13(9):1096–101.
11. Haase VH. Regulation of erythropoiesis by hypoxia-inducible factors. *Blood Rev*. 2013;27:41–53.
12. Schmidt PJ, Toran PT, Giannetti AM et al. The transferrin receptor modulates Hfe-dependent regulation of hepcidin. *Cell Met*. 2008;7(3):205-14. doi:10.1016/j.cmet.2007.11.016
13. Goswami T, Andrews NC. Hereditary hemochromatosis protein, HFE interaction with transferrin receptor2, suggests a molecular mechanism for mammalian iron sensing. *J. Biol. Chem*. 2006;281:28494–98.
14. Ganz T, Nemeth E. Hepcidin and iron homeostasis. *Biochim Biophys Acta*. 2012;1823:1434–43.
15. Kawabata H, Fleming RE, Rui D et al. Expression of hepcidin is down regulated in TfR2 mutant mice manifesting a phenotype of hereditary hemochromatosis. *Blood*. 2004;105:378–81.
16. Malyszko J. Hemojuvelin: the hepcidin story continues. *Kidney Blood Press Res*. 2009;32(2):71–76.
17. Klausner RD, Rouault TA, Harford JB. Regulating the fate of mRNA: the control of cellular iron metabolism. *Cell*. 1993;72:19–28.
18. Kawasaki N, Morimoto K, Tanimoto T et al. Control of hemoglobin synthesis in erythroid differentiating K562 cells. I. Role of iron in erythroid cell heme synthesis. *Arch Biochem Biophys*. 1996;328:289–94.
19. Rouault TA, Klausner RD. Iron-sulfur clusters as biosensors of oxidants and iron. *Trends Biochem Sci*. 1996;21:174–77.
20. Muhlenhoff U, Gerber J, Richhardt N et al. Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *EMBO J*. 2003;22:4815–25.
21. Killip S, Bennett JM, Chambers MD. Iron deficiency anemia. *Am Fam Physician*. 2007;75:611–18.
22. Ferri C, Procianoy RS, Silveira RC. Prevalence and risk factors for iron-deficiency anemia in very-low-birth-weight preterm infants at 1 year of corrected age. *J Trop Pediatr*. 2014;60(1):53–60.
23. Oski FA. Iron deficiency in infancy and childhood. *NEJM*. 1993;329(3):190–93.
24. Domellof M. Iron and other micro-nutrient deficiencies in low-birth weight infants. *Nestle Nutrition Institute Workshop Series*. 2013;74:197–206.
25. Clark SB. Iron deficiency anemia: diagnosis and management. *Curr Opin Gastroentero*. 2009;25:122–28.
26. Brumberg, JJ. Chlorotic girls, 1870–1920: a historical perspective on female adolescence. *Child Dev*. 1982;53:1468–77.
27. Joosten E, Ghesquiere B, Linthoudt H et al. Upper and lower gastrointestinal evaluation of elderly inpatients who are iron deficient. *Amer J Med*. 1999;107(1):24–29.
28. Napolitano M, Dolce A, Celenza G et al. Iron-dependent erythropoiesis in women with excessive menstrual blood losses and women with normal menses. *Ann Hematol*. 2014;993(4):557–63.
29. Major A, Mathez-Loic F, Rohling R. The effect of intravenous iron on the reticulocyte response to recombinant human erythropoietin. *Br J Haematol*. 1997;98(2):292–94.
30. Fernandez-Rodriquez AM, Guindeo-Casasus MC, Molero-Labarta T et al. Diagnosis of iron deficiency in chronic renal disease. *Amer J Kidney Dis*. 1999;34(3):508–13.
31. Thompson WG, Meola T, Lipkin M Jr et al. Red cell distribution width, mean corpuscular volume, and transferrin saturation in the diagnosis of iron deficiency. *Arch Intern Med*. 1988;148:2128–30.
32. Braugnara C, Zurakowski D, Dicanzio J et al. Reticulocyte hemoglobin content to diagnose iron deficiency in children. *JAMA*. 1999;281:2247–48.
33. Aarna PV, Austin RD, Mathew P. Pica. *Indian J Dent Res*. 2012;23(3):426-27.
34. Geissler PW, Shulman CE, Prince RJ et al. Geophage, iron status, and anaemia among pregnant women on the coast of Kenya. *Transac Royal Soc Trop Med Hyg*. 1998;92(5):549–53.
35. Idjradinata P, Pollitt E. Reversal of developmental delays in iron-deficient anaemic infants treated with iron. *Lancet*. 1993;341:1–4.
36. Roncagliolo M, Garrido M, Walter T et al. Evidence of altered central nervous system development in infants with iron deficiency anemia at 6 months: delayed maturation of auditory brainstem responses. *Am J Clin Nutri*. 1998;68(3):683–90.

37. Cullen P, Soffker J, Hopfl M, Bremer C et al. Hypochromic red cells and reticulocyte hemoglobin content as markers of iron-deficient-erythropoiesis in patients undergoing chronic haemodialysis. *Nephrol Dial Transplant*. 1999;14(3):659–65.
38. Vreugdenhil G, Baltus CA, van Eijk HG et al. Anaemia of chronic disease: diagnostic significance of erythrocyte and serological parameters in iron deficient rheumatoid arthritis patients. *Br J Rheumatol*. 1990;29:105–10.
39. Van Vranken M. Evaulation of microcytosis. *Amer Fam Physician*. 2010;82(9):1117-22.
40. Kis AM, Carnes M. Detecting iron deficiency in anemic patients with concomitant medical problems. *J Gen Intern Med*. 1998;13(7):455–61.
41. Van den Broek NR, Letsky EA, White SA et al. Iron status in pregnant women: Which measurements are valid? *Br J Haematol*. 1998;103(3):817–24.
42. McKay R, Scott BB. Iron deficiency anemia: How far to investigate? *Gut*. 1993;34:1427–28.
43. Wong SS, Qutishat AS, Lange J et al. Detection of iron-deficiency anemia in hospitalized patients by zinc protoporphyrin. *Clin Chim Acta*. 1996;244(1):91–101.
44. Rettmer Rl, Carlson TH, Origenes ML et al. Zinc protoporphyrin/heme ratio for diagnosis of preanemic iron deficiency. *Pediatrics*. 1999;104(3):e37.
45. Labbe RF, Dewanji A. Iron assessment tests: transferrin receptor vis-à-vis zinc protoporphyrin. *Clin Biochem*. 37(3):165-74.2004.
46. Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuipen MM. Combined use of erythrocyte zinc protoporphyrin and mean corpuscular volume in differentiation of thalassemia from iron deficiency anemia. *Europ J Haematol*. 1998;60(4):245–51.
47. Junca J, Flores A, Roy C et al. Red cell distribution width, free erythrocyte protoporphyrin, and England-Fraser index in the differential diagnosis of microcytosis due to iron deficiency or beta-thalassemia trait: a study of 200 cases of microcytic anemia. *Hematol Pathol*. 1991;5:33–36.
48. Suominen P, Punnonen K, Ragamaki A et al. Serum transferrin receptor and transferrin receptor-ferritin index identify healthy subjects with subclinical iron deficits. *Blood*. 1998;92(8):2934–39.
49. Punnonen K, Irjala K, Rajamaki A. Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency. *Blood*. 1997;89(3):1052–57.
50. Mulherin D, Skelly M, Saunders A et al. The diagnosis of iron deficiency in patients with rheumatoid arthritis and anemia: an algorithm using simple laboratory measures. *J Rheum*. 1996;23(2):237–40.
51. Ahluwalia N. Diagnostic utility of serum transferrin receptors measurement in assessing iron status. *Nutri Rev*. 1998;56(5 Pt1):133–41.
52. Case G. Maintaining iron balance with total-dose infusion of intravenous iron dextran. *Anna J*. 1998;25(1):65–68.
53. Adamson J. Erythropoietin, iron metabolism, and red blood cell production. *Sem Hematol*. 1996;33:5–9.
54. Chang CC, Kass L. Clinical significance of immature reticulocyte fraction determined by automated reticulocyte counting. *Am J Clin Pathol*. 1997;108(1):69–73.
55. Dallman PR, Reeves JD, Driggers DA et al. Diagnosis of iron deficiency: the limitations of laboratory tests in predicting response to iron treatment in 1-year-old infants. *J Pediatr*. 1981;99(3):376–81.
56. Krantz SB. Pathogenesis and treatment of the anemia of chronic disease. *Am J Med Sci*. 1994;307:353–59.
57. Means RT. Clinical application of recombinant erythropoietin in the anemia of chronic disease. *Hematol Oncol Clin No Amer*. 1994;8(5):933–44.
58. Dicato M, Plawny L, Diederich M. Anemia in cancer. *Ann Oncology*. 2010;21(Supplement 7):vii167-72. doi:10.1093/annonc/mdq284.
59. Baer AN, Dessypris EN, Goldwasser E et al. Blunted erythropoietin response to anemia in rheumatoid arthritis. *Br J Haematol*. 1987;66:559–64.
60. Means RT Jr, Krantz SB. Progress in understanding the pathogenesis of the anemia of chronic disease. *Blood*. 1992;80:1639–47.
61. Jain S, Narayan S, Jagdish S et al. Evaluation of serum transferrin receptor and sTfR indices in diagnosing and differentiating iron deficiency anemia from anemia of chronic disease. *Indian J Pediatr*. 2010;77(2):179-83.
62. Bottomley SS, May BK, Cox TC et al. Molecular defects of erythroid 5-aminolevulinate synthase in s-linked sideroblastic anemia. *J Bioenerg Biomembr*. 1995;27(2):161–68.
63. May A, Bishop DF. The molecular biology and pyridoxine responsiveness of x-linked sideroblastic anemia. *Haematologica*. 1998;83:56–70.
64. Furuyama K, Fujita H, Nagi T et al. Pyridoxine refractory x-linked sideroblastic anemia caused by a point mutation in the erythroid 5-aminolevulinate synthase gene. *Blood*. 1997;90 (2):822–30.
65. Koc S, Harris JW. Sideroblastic anemias: variations on imprecision in diagnostic criteria; proposal for an extended classification of sideroblastic anemias. *Am J Hematol*. 1998;57:1–6.
66. Landrigan PJ, Todd AC. Lead poisoning. *West J Med*. 1994;161:153–59.
67. CDC. Adult blood lead level epidemiology and surveillance –United States 2008-09. *MMWR*. July 1, 2011;60(25):841–45 (available at www.cdc.gov/mmwr/preview/mmwrhtml/mm6025a2.htm; accessed August 6, 2012).
68. CDC. CDC response to Advisory Committee on childhood lead poisoning prevention recommendations in "low level lead exposure harms children: a renewal call of primary prevention." 2012;61(20) (available at www.cdc.gov/nceh/lead/ACCLPP/CDC_Response_lead_exposure_recs.pdf; accessed September 6, 2013).
69. Bhambhani K, Aronow R. Lead poisoning and thalassemia trait or iron deficiency: the value of the red blood cell distribution width. *Am J Dis Child*. 1990;144:1231–33.
70. Carraccio CL, Bergman GE, Daley BP. Combined iron deficiency and lead poisoning in children: effect on FEP levels. *Clin Pediatr*. 1987;26:644–47.
71. Clark M, Royal J, Seeler, R. Interaction of iron deficiency and lead and the hematologic findings in children with severe lead poisoning. *Pediatrics*. 1988;81:247–54.
72. Rainey PM. Lead: a heavy burden for small children. *Clin Chem News*. 1992;18(6):37.
73. Savage D, Lindenbaum J. Anemia in alcoholics. *Medicine*. 1986;5:322–39.
74. Cotter PD, May A, Li L et al. Four new mutations in the erythroid-specific 5-aminoluvulinate synthase (ALSA2) gene causing x-linked sideroblastic anemia: increased pyridoxine responsiveness after removal of iron overload by phlebotomy and coinheritance of hereditary hemochromatosis. *Blood*. 1999;93(5):1757–69.
75. Brittenham, GM. Disorders of iron metabolism: iron deficiency and iron overload. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice. 5th ed*. New York: Churchill Livingstone; 2009 (available at www.mdconsult.com/books/page.do?eid=4-u1.0-B978-0-443-06715-0..X5001-8&isbn=978-0-443-06715-0&uniqId=350057742-2#4-u1.0-B978-0-443-06715-0..X5001-8--TOP; accessed September 6, 2013).
76. Feder JN, Gnirke A, Thomas W. A novel class-I1 like gene is mutated in patients with hereditary hemochromatosis. *Nature Genet*. 1996;13:399–408.
77. Kasvosve I. Effect of ferroportin polymorphism on iron homeostasis and infections. *Clin Chim Acta*. 2013;416;20–25.
78. H(+)-coupled divalent metal-io transporter-1: functional properties, physiological roles and therapeutics. *Curr Top Membr*. 2012;70:169-214.
79. Cogswell ME, Burke W, McDonnell SM et al. Screening for hemochromatosis: a public health perspective. *Am J Prev Med*. 1999;16(2):134–40.
80. Olynyk JK. Hereditary haemochromatosis: diagnosis and management in the gene era. *Liver*. 1999;19:73–80.
81. Fletcher LM, Halliday JW, Powell LW. Interrelationships of alcohol and iron in liver disease with particular reference to the iron-binding proteins, ferritin, and transferrin. *J Gastroentero Hepat*. 1999;14:202–14.
82. Adams PC, Kertesz AE, Valberg LS. Rate of iron reaccumulation following iron depletion in hereditary hemochromatosis: implications for venesection therapy. *J Clin Gastroenterol*. 1993;16(3):207–10.
83. DeVerneuil H, Ged C, Bouleihfar S et al.Porphyrias: animal models and prospects for cellular and gene therapy. *J Bioenerg Biomembr*. 1995;27(2):239–48.
84. Mascaro JM. Porphyrias in children. *Pediatr Dermatol*. 1992;9:371–72.
85. Fuller SJ, Wiley JS. Heme biosynthesis and its disorders. In: Hoffman R, Benz EJ, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. New York: Elsevier; 2013:457–72 (available at https:www-clinicalkey-com; accessed June 18, 2014).



13 Hemoglobinopathies: Qualitative Defects

REBECCA J. LAUDICINA, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *hemoglobinopathy*.
2. Explain the basis of defects resulting in the production of variant hemoglobins.
3. Explain the basis of the hemoglobin electrophoresis method in identifying variant hemoglobins.
4. Describe the epidemiology of sickle cell anemia (SCA) and other hemoglobinopathies.
5. Identify the globin chain defects causing SCA, hemoglobin C (HbC) disease, and hemoglobin E (HbE) disease.
6. Associate laboratory analyses with their use in detecting and identifying hemoglobinopathies.
7. Recognize and identify abnormal laboratory test results, including peripheral blood findings and screening and confirmatory tests, typically associated with homozygous and heterozygous conditions involving sickle hemoglobin (HbS), HbC, HbD, and HbE and compound heterozygous conditions involving HbS and other variant hemoglobins.
8. List major clinical findings typically associated with the hemoglobinopathies listed in Objective 7.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Compare the synthesis and concentration of variant hemoglobins in homozygous and heterozygous conditions.
2. Compare the prevalence of hemoglobins S, C, D, and E.
3. Compare and contrast the pathophysiology of common hemoglobin variants in terms of altered solubility, function, and stability.
4. Analyze the structure of the hemoglobin molecule in sickle cell anemia (SCA) and relate it to the pathophysiology of the disease.
5. Contrast clinical findings in persons who are homozygous and heterozygous for hemoglobins S, C, D, and E and in those who have combined heterozygosities for these variant hemoglobins.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

6. Identify and explain the basis of current therapies for SCA.
7. Evaluate and interpret mobility patterns obtained on cellulose acetate and citrate agar gel hemoglobin electrophoresis when structurally abnormal hemoglobins are present.
8. Select, evaluate, and interpret tests used in detecting and identifying abnormal hemoglobins.
9. Design a laboratory-testing algorithm for optimizing tests used in detecting and identifying variant hemoglobins.
10. Evaluate laboratory test results and medical history of a clinical case for a patient with a hemoglobinopathy and suggest a possible diagnosis.
11. Explain the physiologic abnormality resulting in unstable hemoglobins, methemoglobinemia, and hemoglobin variants with increased or decreased oxygen affinity.
12. Interpret laboratory findings associated with the disorders in Objective 11.

Key Terms

Aplastic crisis
Autosplenectomy
Compound heterozygote
Hemoglobin electrophoresis
Hemoglobinopathy
Irreversibly sickled cell (ISC)
Ischemic
Methemoglobinemia
Sequestration crisis
Thalassemia
Vaso-occlusive crisis

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe erythrocyte metabolism and erythrocyte destruction. (Chapter 5)
- Describe the structure and function of hemoglobin; list variant hemoglobins. (Chapter 6)
- Name and describe basic laboratory procedures used to screen for and assess anemia. (Chapters 10, 11, 37)
- Recognize abnormal values and results for basic hematologic procedures. (Chapters 10, 11, 37)
- Describe the classification systems of anemias. (Chapter 11)

Level II

- Explain the genetic control of globin chain synthesis. (Chapter 6)
- Describe the ontogeny of hemoglobin types. (Chapter 6)

CASE STUDY

We will refer to this case throughout the chapter.

Shane, a 16-year-old African American male with a previously diagnosed hemoglobinopathy, was admitted to the hospital complaining of severe pain in his knees and back. Two of his four siblings have the same disorder. He has been admitted to the hospital on numerous occasions throughout his life for complications of his disease. Physical examination reveals a thin male in acute distress, complaining of severe pain. A head, eyes, ears, nose, and throat (HEENT) exam is positive for corkscrew vessels of the schlerae, schleral icterus, and small, ill-defined, mobile (shotty) cervical lymph nodes. Abdominal exam revealed no splenomegaly, hepatomegaly, tenderness, or masses. Vital signs included temperature 37.8°C, blood pressure 95/70, and pulse 82. Blood was drawn for laboratory tests, and a chest radiograph and MRI of the head were ordered.

Consider whether the patient's current condition is likely to be related to his previous diagnosis and what the laboratory's role is at this time.

OVERVIEW

When hemoglobin's molecular structure is altered, the molecule's function, stability, and/or solubility can change, often resulting in anemia and other clinical consequences. Laboratory screening tests for hemoglobin variants are based on the altered characteristics of the hemoglobin molecule. If screening tests are abnormal, reflex tests are required to confirm the presence of a variant hemoglobin. The laboratory's role is not limited to detecting variant hemoglobins but extends to monitoring a patient's condition over the course of an illness and during treatment. This chapter discusses the most common variant hemoglobins including epidemiology, pathophysiology, clinical findings, laboratory findings, and treatment. Emphasis is on the correlation of clinical history and symptoms with laboratory tests and interpretation of test results.

INTRODUCTION

Clinical diseases that result from a genetically determined abnormality of the structure or synthesis of the hemoglobin molecule are called **hemoglobinopathies**. The abnormality is associated with the globin chains; the heme portion of the molecule is normal. The globin abnormality can be either a qualitative defect

in the globin chain (structural abnormality) or a quantitative defect in globin synthesis.

Qualitatively abnormal hemoglobin molecules arise from genetic mutations in the coding region of a globin gene, resulting in amino acid deletions or substitutions in the globin protein chain. These mutations cause structural variation in one of the globin chain classes (structural hemoglobin variants). The nomenclature of these disorders is discussed in the later section "Nomenclature." The most common clinical disorder of this type of mutation is sickle cell anemia.

The quantitative globin disorders result from various genetic defects that reduce synthesis of structurally normal globin chains. The quantitative disorders are known collectively as **thalassemias**.

Clinical disorders are also associated with combination defects involving both structural defects and quantitative deficiencies of hemoglobin. Compound heterozygotes possess two different abnormal alleles of a gene coding for globin chains. An example is thalassemia in combination with sickle cell (Chapter 14).

As a result of the globin chain defects, hemoglobinopathies can be associated with a chronic hemolytic anemia or can be asymptomatic. Clinical expression of the hemoglobinopathy varies, depending on the class of globin chain involved (α, β, δ, or γ), the severity of hemolysis, and the compensatory production of other normal globin chains. Some of the hemoglobinopathies produce no clinical signs or symptoms of disease and are identified only through population studies specifically designed to reveal "silent" carriers. As discovery of silent carriers increases, the incidence of these genetic disorders is proving to be much higher than originally thought.

Hemoglobinopathies are believed to be the most common lethal hereditary diseases in humans.[1] More than 330,000 infants worldwide are born each year with inherited hemoglobin disorders such as sickle cell and thalassemia.[2] Many affected children live in low-income countries, and many die before age 5.

Hemoglobinopathies are found worldwide but occur most commonly in African blacks and ethnic groups from the Mediterranean basin and Southeast Asia. The geographic locations where the quantitative and qualitative hemoglobin disorders are found frequently overlap; thus, it is not uncommon for individuals to have both a structural hemoglobin variant and a form of thalassemia. This could partly explain the extreme variation in clinical findings associated with hemoglobinopathies.

This chapter discusses the structural hemoglobin variants and Chapter 14 discusses the thalassemias and combination disorders.

STRUCTURAL HEMOGLOBIN VARIANTS

The largest group of hemoglobinopathies results from an inherited structural change in one of the globin chains; however, rate of synthesis of the abnormal chain usually is not significantly impaired. Any of the globin chain classes, α, β, δ, or γ, can be affected.

Sickle cell anemia (SCA), the most common structural hemoglobin variant, was reported by James Herrick of Chicago in 1910.[3] He described the typical crescent-shaped sickled erythrocytes in a young black student from the West Indies. Following this initial report, additional cases of the disease were described, and the clinical pattern of SCA was established. The pathophysiologic aspects of the disease, however, remained a mystery until Linus Pauling in 1949 discovered the altered electrophoretic mobility of the hemoglobin in patients with sickle cell disease.[4] The molecule's altered electrical charge was ascribed to a molecular abnormality of the globin chain.

More than 1100 abnormal hemoglobins have since been identified.[5] A molecular database of abnormal hemoglobins can be accessed at http://globin.cse.psu.edu/globin/hbvar/menu.html. The number of identified β-chain mutations exceeds the number identified for α-, δ-, or γ-chains (Table 13-1 ★). Part of the explanation for this distribution likely resides in the genetics and phenotypic expression of the globin gene loci. Hemoglobin F (γ-chain) is expressed to a significant extent only during fetal development; therefore, variant F hemoglobins are unlikely to be detectable after 3–6 months of age. Mutations affecting the γ-chain locus with the potential to produce a significant clinical abnormality would likely cause fetal death. At birth and in the first few months of life, as β-chain synthesis becomes predominant, residual abnormal HbF is masked by the increasing concentration of HbA in children carrying mutations of the γ-chain, producing milder clinical presentations.

Because HbA_2 (δ-chain) is usually a minor component of the total hemoglobin in adults, HbA_2 variants are also unlikely to cause clinical complications and are discovered "accidentally" during laboratory evaluation for other purposes.

Two copies of the α-chain gene are found on each of the number 16 chromosomes, resulting in a total of four genes. Thus, a mutation of a single α-locus resulting in a variant α-chain-containing hemoglobin produces only a small amount of the abnormal hemoglobin and is less likely to cause clinically significant disease.

The β-gene, which is found in a single copy on each chromosome 11, is most likely to be associated with a clinical phenotype when mutated because the β-globin chain is a component of HbA, the major adult hemoglobin.

Identification of Hemoglobin Variants

Most structural hemoglobin variants result from a single amino acid substitution or deletion in the globin polypeptide chain. The majority of structural variants result in no clinical or hematologic abnormality and have been discovered only by population studies or family studies. Mutations result in clinical manifestations only when the solubility, stability, or function (oxygen affinity) of the hemoglobin molecule is altered. These phenotypic variants produce both clinical and hematologic abnormalities of varying severity, depending on the nature and site of the mutation. Laboratory tests designed to detect and identify hemoglobin variants are based on the molecule's altered structure or function.

★ **TABLE 13-1** Structural Variants of Globin Genes

Globin Polypeptide Gene	Number of Variants
α1- and/or α2-globin gene(s)	424
β-globin gene	571
δ-globin gene	69
Aγ-globin gene	38
Gγ-globin gene	58

From globin.cse.psu.edu/globin/hbvar/menu.html (accessed March 14, 2014).

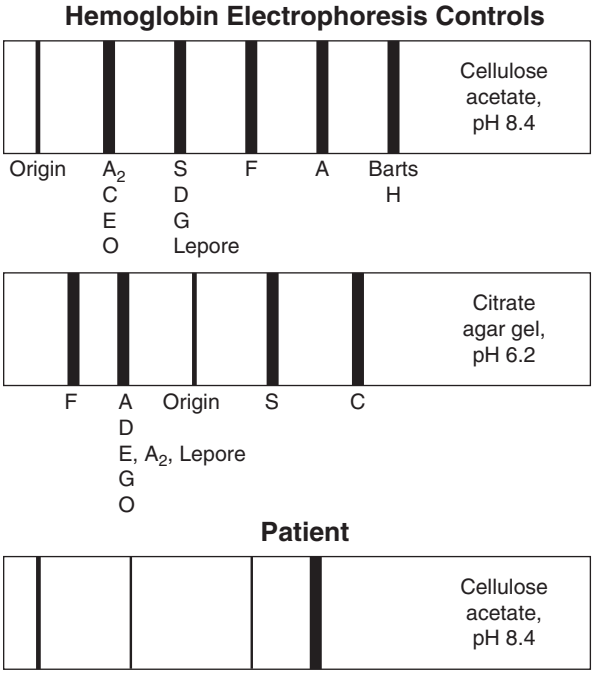
Methods of Analysis

Hemoglobin carries an electrical charge resulting from the presence of ionized carboxyl (COO^-) and protonated (H^+) amino (NH_3^+) groups. The type (net positive, net negative) and strength of the charge depend on both the amino acid sequence of globin chains in the hemoglobin molecule and the pH of the surrounding medium. Many amino acid substitutions alter the molecule's electrophoretic charge, enabling the detection of a structural hemoglobin variant by **hemoglobin electrophoresis**. Some substitutions can cause identical changes in the net charge of the molecule; thus, two different mutant hemoglobins can have identical electrophoretic mobility. Other substitutions do not alter the charge of the globin chain, and the variant hemoglobin migrates identical to the "normal" globin chain. By varying the medium and pH of the procedure, many clinically significant hemoglobins can be detected and identified. Methods for performing hemoglobin electrophoresis, examples of electrophoretic patterns, and more complete discussions of the tests that follow are included in Chapter 37.

The most common clinically symptomatic hemoglobinopathies involve abnormalities of the β-globin chain, resulting in a decrease or absence of HbA ($\alpha_2\beta_2$) and sometimes an increase in HbF ($\alpha_2\gamma_2$) and/or HbA_2 ($\alpha_2\delta_2$). A band representing the mutant hemoglobin may also be present. Typically, an elevation in HbF and/or HbA_2 is the clue to the presence of a hemoglobinopathy, although HbF can be elevated in other hematologic disorders as well. HbF concentrations >10% can be measured by electrophoresis and densitometry. Smaller but significant increases in HbF can be measured more accurately by alkali denaturation and other methods. HbF distribution among the erythrocyte population is evaluated by acid elution tests. These tests are based on the fact that HbF is more resistant to alkali and acid treatment than other hemoglobins.

Results of laboratory tests provide essential information when a hemoglobinopathy is suspected. The hemoglobin concentration from the CBC indicates whether anemia is present. RDW and erythrocyte indices should be evaluated to help distinguish hemoglobinopathies from thalassemia, which usually causes a microcytic, hypochromic anemia. A review of the blood smear can reveal specific poikilocytes, such as sickle cells.

An important next step is to detect hemoglobin variants and quantify hemoglobins A_2 and F.[6] Several options, ranging from traditional electrophoresis on solid media to newer methods, are available; many laboratories use a combination of tests. Figure 13-1 ■ shows the electrophoretic mobility of normal and abnormal hemoglobins using cellulose acetate (pH 8.4) and citrate agar gel (pH 6.2). Some laboratories are using advanced testing methods such as isoelectric focusing (IEF), high-performance liquid chromatography (HPLC), or capillary electrophoresis (CE).[7] These laboratory methods are suitable for testing large numbers of individuals for hemoglobinopathies and offer improved resolution and identification of certain hemoglobin variants over results obtained with alkaline electrophoresis.[8] HPLC has the advantage over electrophoresis of quantifying low concentrations of HbA_2 and HbF.[7] All states in the United States mandate newborn screening programs for sickle cell disease,[9] often using HPLC or mass spectrometry methods that are appropriate for high-volume testing. Unfortunately, these methods may be unavailable for testing residents of developing and low-income countries.



■ **FIGURE 13-1** Electrophoretic patterns of hemoglobins. The top and middle patterns are controls for electrophoresis by cellulose acetate at pH 8.4 and citrate agar gel at pH 6.2 showing the mobility patterns for normal and abnormal hemoglobins. Note that some hemoglobins that move together on cellulose acetate (pH 8.4) (e.g., HbS, D, G) can be separated on citrate agar gel (pH 6.2). The bottom pattern shows an electrophoretic pattern of a patient with normal hemoglobin using cellulose acetate at pH 8.4.

Other traditional tests for abnormal hemoglobins are based on altered physical properties of the structural variants. These include solubility tests, heat precipitation tests, and tests for Heinz bodies. The uses of these methods are included in the following discussion of the corresponding specific structural variants. Procedures are included in Chapter 37.

Techniques are also available to identify the specific molecular defect of hemoglobin disorders.[10] These techniques are discussed in Chapter 42. The polymerase chain reaction (PCR) has been incorporated into diagnostic procedures for identifying point mutations because it enhances sensitivity and reduces the amount of DNA and time required for analysis. Prenatal diagnosis can be carried out in the first trimester of pregnancy using DNA obtained from chorionic villus sampling. In cases of hemoglobinopathies caused by known common mutations, such as sickle cell anemia, the fetal DNA can be analyzed directly. If prenatal diagnosis is desired when the exact mutation is not known, the parents' DNA could be analyzed first (e.g., restriction fragment length polymorphism [RFLP]) to help identify the presence of a mutation (Chapter 42).

☑ CHECKPOINT 13-1

Why is it not possible for all structural hemoglobin variants to be identified by hemoglobin electrophoresis?

CASE STUDY (continued from page 232)

1. Identify a laboratory test needed to determine Shane's hemoglobinopathy.

Nomenclature

The first abnormal hemoglobin discovered was called *hemoglobin S* (*HbS*) because it was associated with crescent (sickle)-shaped erythrocytes (S for sickle). Subsequently, other hemoglobin variants were discovered and were given successive letters of the alphabet according to electrophoretic mobility beginning with the letter C. The letter *A* was already being used to describe the normal adult hemoglobin, HbA. The letter *B* was not used to avoid confusion with the ABO blood group system. The letter *F* had been designated to describe fetal hemoglobin, HbF. The letter *M* was given to those hemoglobins that tended to form methemoglobin (HbM).

As more variants were discovered, it was recognized that the alphabetical system was not sufficient and a different nomenclature system was needed. Thus, subsequent hemoglobins were given common names according to the geographic area in which they were discovered (e.g., Hb Ft. Worth). It also became apparent that some variants with the same letter designation (same electrophoretic mobility) had different structural variations. If the hemoglobin has the electrophoretic mobility of a previously lettered hemoglobin, that letter is used in addition to the geographic area (e.g., HbG Honolulu).

A standardized hemoglobin nomenclature has been recommended for use. All variants should be given a scientific designation as well as a common name. The scientific designation includes the following: (1) the mutated globin chain, (2) the position of the affected amino acid, (3) the helical position of the mutation, and (4) the amino acid substitution. (If the mutation affects amino acids between helices, the number of the amino acid and the letters of the two bracketing helices are used.) For example, HbS is designated β6 (A3) Glu $\rightarrow$ Val. The mutation is in the β-chain affecting the amino acid in the sixth position of the completed polypeptide located in the A3 helix position. The amino acid valine is substituted for glutamic acid. Hemoglobins with amino acid deletions include the word *missing* after the amino acid and helix designation (e.g., β56–59 [D7–E3] missing). The advantage of the helical designation is that amino acid substitutions in the same helix can lead to similar functional and structural alterations of the hemoglobin molecule, allowing a better understanding of the clinical manifestations of each.

Not all globin chain mutations cause symptoms of disease; thus, many go undetected. Only those that cause clinical symptoms are likely to be brought to a physician's attention. If an individual is homozygous for the gene coding for a structural β-globin mutant, no HbA is produced, and the term *disease* or *anemia* is used to describe the specific disorder (e.g., sickle cell anemia). If one of the genes coding for the β-chain is normal and the other β-gene codes for a structural variant, both HbA and the abnormal hemoglobin are produced, and the word *trait* is used to describe the heterozygous disorder (e.g., sickle cell trait).

With the most common β-chain hemoglobin variants (HbS, HbC), the abnormal hemoglobin usually accounts for $<$50% of the total hemoglobin in the trait form, whereas in the homozygous state of disease, the abnormal hemoglobin usually constitutes 90–95% of the total hemoglobin. This is explained by the effect of the abnormal globin chain on the formation of the hemoglobin tetramers. The normal α-chain has a net positive charge; the normal β-chain (β^A) has a negative charge, and $\alpha\beta$ dimers form initially through positive-negative electrostatic interactions. β-chain mutants with a lesser negative charge than β^A form $\alpha\beta$ dimers more slowly than do β^A (and, conversely mutations that increased the negative charge of the β-globin chain would form $\alpha\beta$ dimers more rapidly). Both β^S and β^C mutations cause a net reduction of the β-chain negative charge and form $\alpha\beta^S$ or $\alpha\beta^C$ dimers more slowly than $\alpha\beta^A$ dimers. As a result, heterozygotes have ~60% HbA and ~35–40% HbS or HbC. If HbS or HbC constitutes $>$50% of the total hemoglobin in a heterozygote, the patient could have inherited two different abnormal hemoglobin genes (**compound heterozygote**) or a form of thalassemia in combination with the structural hemoglobin variant (Chapter 14).

☑ CHECKPOINT 13-2

What does the term *silent carrier* mean when referring to a hemoglobinopathy?

CASE STUDY

Results of hemoglobin electrophoresis were 90% HbS, 9% HbF, and 1% HbA_2.

2. What is the abnormal hemoglobin causing Shane's disease?
3. Is Shane heterozygous or homozygous for the disorder?
4. What is this disorder called?

Pathophysiology

The structural hemoglobin variants cause symptoms if the amino acid substitution occurs at a critical site within the molecule. Mutations that cause clinical signs of disease affect the solubility, function (oxygen-affinity), and/or stability of the hemoglobin molecule.

Altered Solubility

If a nonpolar amino acid is substituted for a polar residue near the molecule's surface, the solubility of the hemoglobin molecule can be affected. Hemoglobin S and hemoglobin C are examples of this type of substitution. In the deoxygenated state, the HbS molecule polymerizes into insoluble, rigid aggregates. The majority of surface substitutions, however, do not affect the tertiary structure, heme function, or subunit interactions and are therefore innocuous.

Altered Function

Some amino acid substitutions can affect the oxygen affinity of hemoglobin by stabilizing heme iron in the ferric state, producing methemoglobin, which cannot combine with oxygen (Chapter 6). Hemoglobins M and Chesapeake are examples. Mutations within the

subunit interface, $\alpha_1\beta_2$, can affect the allosteric properties of the molecule, leading to increased or decreased oxygen affinity. Considerable movement occurs at the $\alpha_1\beta_2$ contact region on oxygenation, which triggers these allosteric interactions (Chapter 6). High oxygen-affinity hemoglobin variants produce congenital erythrocytosis whereas decreased oxygen-affinity variants produce pseudoanemia and cyanosis.

Altered Stability

Amino acid substitutions that reduce the stability of the hemoglobin tetramer result in unstable hemoglobins. The mutations usually disrupt hydrogen bonding or hydrophobic interactions that retain the heme component within the heme-binding pocket of the globin chain or that hold the tetramer together. The result is a weakening of the binding of heme to globin and detachment of the heme or the disruption of the integrity of hemoglobin's tetrameric structure. Consequently, hemoglobin denatures, aggregates, and precipitates as Heinz bodies. Clinically, the unstable hemoglobin variants are sometimes known as *congenital Heinz body hemolytic anemias*. In addition to altering the molecule's stability, disruption of normal conformation also can affect the molecule's function.

☑ CHECKPOINT 13-3

The mutation in HbJ-Capetown, α92, Arg → Gln, stabilizes hemoglobin in the R state (Chapter 6). What functional effect does this have on the hemoglobin molecule?

SICKLE CELL ANEMIA

Worldwide, sickle cell anemia is the most common symptomatic hemoglobinopathy with greatest prevalence in tropical Africa (Table 13-2 ★). Gene frequency in equatorial Africa can exceed 20%. The sickle cell gene is also common in areas around the Mediterranean, the Middle East, India, Nepal, and in geographic regions in which there has been migration from endemic areas, such as North, Central, and South America.[11] Sickle cell anemia occurs in 0.3–1.3%, and sickle cell trait occurs in 8–10% of African Americans.

It is interesting to note that geographic areas with the highest frequency of sickle cell genes are also areas where infection with *Plasmodium falciparum* is common. This correlation strongly suggests that HbS in heterozygotes confers a selective advantage against fatal malarial infections, resulting in an increase in the gene frequency. Children with sickle cell trait are infected with the malarial parasite, but the parasite counts remain low. It has been suggested that resistance to malaria occurs because parasitized cells sickle more readily, leading to sequestration and phagocytosis of the infected cell by the spleen. Other as yet undefined factors could also contribute to reduced malarial susceptibility in individuals with HbS.[12] Epidemiological data suggest there could be similar selective advantages to HbE and HbC.[11] Molecular evidence indicates that the identical sickle mutation arose independently in these geographic areas at least five times.

Pathophysiology

Sickle cell anemia is caused by a mutation (GAG → GTG conversion) in the *HBB* gene (hemoglobin β gene). This mutation results in the substitution of nonpolar valine for polar glutamic acid at the sixth amino acid position in the A3 helix of the β-chain—β6 (A3) Glu → Val (see the previous "Nomenclature" section for explanation)—and produces the mutant hemoglobin, HbS. The amino acid substitution is on the surface of the molecule, producing a net decrease in negative charge; hence, it changes the molecule's electrophoretic mobility. The solubility of HbS in the deoxygenated state is markedly reduced, producing a tendency for deoxyhemoglobin S molecules to polymerize into rigid aggregates. The cells may assume a crescent shape, depending on the extent of polymerization of the HbS molecules. Polymerization is reversible on reoxygenation.

Polymerization is time dependent. A time delay occurs between deoxygenation and the formation of a significant amount of HbS polymers. The development of significant polymerization and red cell distortion takes ~2–4 minutes. The delay time for polymerization is important in considering the overall clinical consequences of HbS. Even though most red cells contain some sickle hemoglobin polymer at the oxygen concentration in venous blood, the majority of the cells do not sickle during their journey through the circulation because they reach the lungs and are reoxygenated before significant polymerization and cell distortion occur. The length of delay depends highly on temperature, pH, ionic strength, and oxygen tension in the cell's environment. Hypoxia, acidosis, hypertonicity, and temperatures higher than 37°C promote deoxygenation and the formation of HbS polymers. The spleen, kidney, retina, and bone marrow provide a sufficiently hypoxic, acidotic, and hypertonic microenvironment to promote HbS polymerization and sickling.

★ **TABLE 13-2** Summary of the Most Common Hemoglobin Variants

Hb	Peripheral Blood, Homozygotes	Hb Present on Electrophoresis	Mutation Lys	Geographic Distribution
HbS	Normocytic, normochromic anemia; reticulocytosis; poikilocytosis with sickled and boat-shaped cells	Homozygous: HbS, F, A_2 Heterozygous: HbA, S, F, A_2	β6(A3) Glu → Val	Tropical Africa and Mediterranean areas, Middle East, India, Nepal
HbE	Microcytic, hypochromic anemia; target cells	Homozygous: HbE, F, A_2 Heterozygous: HbA, E, F, A_2	β26(B8) Glu → Lys	Burma, Thailand, Cambodia, Malaysia, Indonesia
HbC	Normocytic, normochromic anemia with reticulocytosis; poikilocytosis with folded, irregularly contracted cells; target cells	Homozygous: HbC, F, A_2 Heterozygous: HbA, C, F, A_2	β6(A3) Glu → Lys	West Africa

Sickling (delay time) also depends on intracellular hemoglobin composition (the proportion of HbA, HbS, HbA_2, and HbF present) as well as total hemoglobin concentration (MCHC). Non-S hemoglobins increase the delay time for polymerization, presumably by interfering with the HbS polymerization process. Altering erythrocyte contents by increasing the concentration of HbF forms the basis of an important treatment for SCA. The delay time is also inversely related to the total hemoglobin concentration. The more concentrated the hemoglobin solution is within the cell (the higher the MCHC), the shorter is the delay time and the greater is the potential for HbS aggregates to form. Using this concept, attempts are made to treat the disease by hydrating the cells, which would decrease the MCHC and prevent sickling.

Polymerization of deoxyhemoglobin S begins when the oxygen saturation of hemoglobin falls below 85% and is generally complete at about 38% oxygen saturation. The HbS aggregates cause the erythrocyte to become rigid and less deformable. With repeated cycles of deoxygenation, polymerization, and sickling, disruption of cation homeostasis occurs, resulting in an increase in intracellular Ca^{++} and loss of K^{+} and water from the cell. This cellular dehydration increases the MCHC, predisposing the cell to sickling on subsequent deoxygenation. In addition, the increased MCHC is associated with an increase in cytoplasmic viscosity and a decrease in cell deformability. HbS is also prone to oxidation. Oxidation of membrane proteins and lipids weakens critical skeletal associations. Repeated sickling tends to decouple the lipid bilayer from the membrane skeleton, and loss of membrane phospholipid asymmetry occurs. The aggregates also damage the erythrocyte membrane directly, and with the weakened cytoskeleton, the cell's fragility increases.

Irreversibly Sickled Cells

The sickled erythrocyte can return to a normal biconcave shape upon reoxygenation of the hemoglobin; however, as described above, with repeated cycles of sickling, the erythrocyte membrane undergoes changes that cause it to become leaky and rigid. After repeated sickling episodes, the cells become **irreversibly sickled cells (ISCs)**, which are locked in a sickle shape whether oxygenated or deoxygenated (i.e., regardless of polymerization state of the hemoglobin molecules). This is due to abnormal interactions of red cell skeletal proteins, most likely caused by oxidative damage and increased Ca^{++} concentrations. The ISC is the result of permanent alterations of the submembrane skeletal lattice.[13] From 5–50% of the circulating erythrocytes in sickle cell anemia are ISCs. This varies from person to person but is relatively constant for a given individual. The ISCs have a very high MCHC and a low MCV. They are ovoid or boat shaped with a smooth outline and lack the spicules characteristic of deoxygenated sickled cells.

The ISCs are removed by mononuclear phagocytes in the spleen, liver, or bone marrow. ISCs account for most, if not all, of the sickle forms on a peripheral blood smear (most reversible sickle cells regain a discocyte shape when the blood is exposed to air while making the slide). The ISCs can also initiate or increase the severity of vaso-occlusive crises because of impaired cell deformability and increased cell adherence to vascular endothelium. The mechanism for this interaction between sickled cells and the endothelium is unclear but could be related to changes in the surface properties of sickled cells and endothelial cells as well as plasma factors.[14,15]

Oxygen Affinity of HbS

The oxygen affinity of HbS differs from that of HbA, resulting in important physiologic changes in vivo. HbS has decreased oxygen affinity, and the 2,3-BPG level of homozygotes is increased. This decreased oxygen affinity, depicted by a shift to the right in the oxygen dissociation curve, facilitates the release of more oxygen to the tissues. However, this phenomenon increases the concentration of deoxyhemoglobin S, promoting the formation of sickle cells.

Red Blood Cell (RBC) Destruction

The primary cause of anemia in sickle cell anemia is extravascular hemolysis. Erythrocyte survival depends on intracellular HbF concentration and degree of membrane damage.[16] Changes in the erythrocyte membrane resulting in increased fragility coupled with Heinz body formation from denatured HbS lead to increased membrane shedding (vesiculation), a decreased cell surface area, and the removal of the cell by the mononuclear phagocyte system.[17] The life span of circulating HbS erythrocytes can decrease to as few as 14 days. The sluggish blood flow and the hypoglycemic, hypoxic environment of the spleen promotes HbS polymerization and sickling, further slowing blood circulation in the splenic cords and enhancing phagocytosis of erythrocytes containing HbS. Eventually, however, the spleen loses its functional capacity as repeated **ischemic** crises (see "Vaso-Occlusive Crisis") lead to splenic tissue necrosis and atrophy. With splenic atrophy, other cells of the mononuclear phagocyte system in the liver and bone marrow take over the destruction of these abnormal cells.

Clinical Findings

The first clinical signs of sickle cell anemia appear at about 6 months of age when the concentration of HbS predominates over HbF. Clinical manifestations result from chronic hemolytic anemia, vaso-occlusion of the microvasculature, overwhelming infections, and acute splenic sequestration.

Anemia

A moderate to severe chronic anemia as the result of extravascular hemolysis is characteristic of the disease. Gallstones, a complication of any chronic hemolytic disorder, are commonly found due to cholestasis and increased bilirubin turnover. Folate deficiency due to increased erythrocyte turnover can further exacerbate the anemia, producing megaloblastosis (Chapter 15). Over time, iron overload and resultant complications, can develop.

Hemodynamic changes occur in an attempt to compensate for the tissue oxygen deficit; as a result, symptoms of cardiac overload including cardiac hypertrophy, cardiac enlargement, and eventually congestive heart failure are frequent complications of the disease.

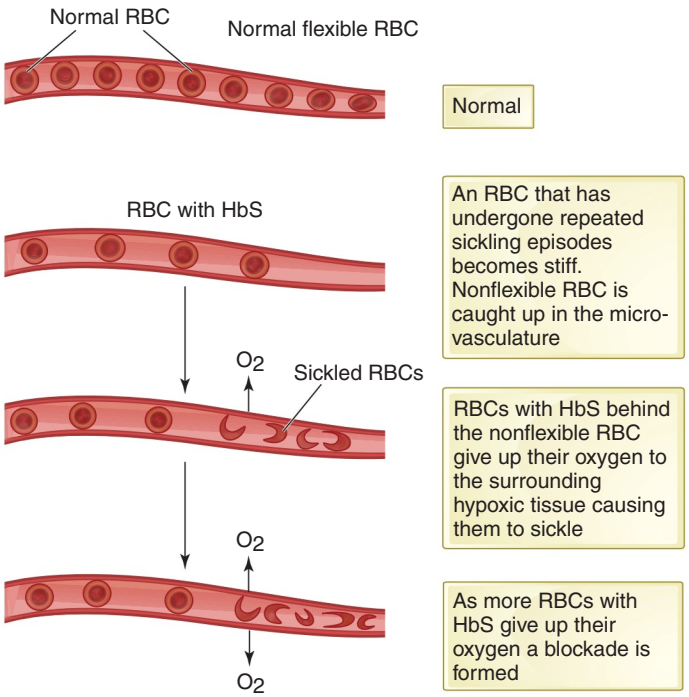
The hyperplastic bone marrow, secondary to chronic hemolysis, is accompanied by bone changes, such as thinning of cortices and a "hair-on-end" appearance in x-rays of the skull. Hyperplasia results from a futile attempt by the marrow to compensate for premature erythrocyte destruction. Conversely, **aplastic crises** can accompany or follow viral, bacterial, and mycoplasmal infections. This temporary cessation of erythropoiesis in the face of chronic hemolysis leads to an acute worsening of the anemia. The aplasia can last from a few days to a week and because of the significantly decreased red cell life span, can induce a catastrophic fall in the hemoglobin concentration.

Increasing evidence suggests that many cases of aplasia occur as a result of infection with human parvovirus B19. Parvovirus also causes a cessation of erythropoiesis in normal individuals, but with a normal RBC life span, normal blood cell production in these individuals is restored before any clinically significant changes in erythrocyte concentration take place.

Vaso-Occlusive Crisis

HbS cells are poorly deformable. Sickled cells have difficulty squeezing through small capillaries, and consequently, the rigid cells tend to aggregate in the microvasculature, increasing vascular stasis. Erythrocytes behind the blockage release their oxygen to the surrounding hypoxic tissue, deoxygenate, polymerize, and sickle, increasing the plug's size (Figure 13-2 ■). Erythrocytes from nearby capillaries are forced to give up more oxygen than they normally would to feed the oxygen-deprived tissue around the blockage. These cells then form rigid aggregates of deoxyhemoglobin S, expanding the blockaded region. If severe, lack of oxygen can cause local tissue necrosis. Vaso-occlusion occurs more often in tissues prone to vascular stasis (spleen, marrow, retina, kidney).

The blockage of the microvasculature by rigid sickled cells accounts for the majority of the clinical signs of sickle cell anemia. The occlusions do not occur continuously but sporadically, causing acute signs of distress. These episodes are called **vaso-occlusive crises** and are the most frequent causes of hospitalization. The crises can be triggered by infection, decreased atmospheric oxygen pressure, dehydration, or slow blood flow, but frequently they occur without any known cause. The occlusions are accompanied by pain, low-grade fever, organ dysfunction, and tissue necrosis. The episodes generally last for 4–5 days and subside spontaneously.



■ **FIGURE 13-2** An illustration showing the way sickle cells block a vessel and precipitate a vaso-occlusive crisis.

Recurrent occlusive episodes can lead to infarctions of tissue of the genitourinary tract, liver, bone, lung, and spleen. The chronic organ damage is accompanied by organ dysfunction. Although splenomegaly is present in early childhood, repeated splenic infarctions eventually result in splenic fibrosis and calcifications (usually by age 4 or 5). This organ damage, secondary to infarction, is known as **autosplenectomy**. As a result, splenomegaly is rare in adults with this disease. Aseptic necrosis of the head of the femur is common. Dactylitis, a painful symmetrical swelling of the hands and feet (hand-foot syndrome) caused by infarction of the metacarpals and metatarsals, is often the first sign of the disease in infants. Recurrent priapism is a characteristic, painful complication that occasionally requires surgical intervention.

The slow flow of blood in occlusive areas can lead to thrombosis. Thrombosis of the cerebral arteries resulting in stroke is common. Magnetic resonance imaging shows evidence of subclinical cerebral infarction in 20–30% of children with sickle cell anemia. If the arterioles of the eye are affected, blindness can occur. Chronic leg ulcers, found also in other hemolytic anemias, can occur at any age. The ulcers appear without any known injury. These painful sores do not readily respond to treatment and can take months to heal.

Placental infarctions in pregnant women with sickle cell disease can be a hazard to the fetus. Maternal anemia often becomes more severe during pregnancy. In addition, other clinical findings can be exacerbated during pregnancy, endangering the life of both the mother and the fetus.

Bacterial Infection

Overwhelming bacterial infection is a common cause of death in young patients, and fever is treated as a medical emergency. The risk of septicemia from encapsulated microorganisms, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, is extremely high in children with SCA who have not received vaccines against these organisms. Bacterial pneumonia is the most common infection, but meningitis is also prevalent. The reasons for this increased susceptibility to infection are not fully understood but could be related to functional asplenia, impaired opsonization, and abnormal complement activation.[18] The spleen is particularly important for host defense in the young. Significant impairment of in vivo neutrophil adherence to vascular endothelium in HbS disease also occurs. This could prevent neutrophils from rapidly relocating to areas of inflammation.[18] Prophylactic penicillin is given to children with sickle cell anemia beginning at age three months to reduce morbidity and mortality from infection, but compliance with therapy may not be optimal. The pneumococcal, meningococcal, and *H. influenzae* type B vaccines protect patients from infections with *S. pneumoniae*, *Neisseria meningitidis*, and *H. influenzae*, reducing the incidence of infection. Children with sickle cell anemia are also routinely immunized for hepatitis B and seasonal influenza.

☑ CHECKPOINT 13-4

Why do newborns with sickle cell anemia not experience episodes of vaso-occlusive crisis?

Acute Splenic Sequestration

In young children sudden splenic pooling of sickled erythrocytes (**sequestration crisis**) can cause a massive decrease in erythrocyte mass within a few hours. Thrombocytopenia can also occur. Hypovolemia and shock follow. At one time, splenic sequestration was the leading cause of death in infants with sickle cell anemia. Early diagnosis, instruction of parents in detecting an enlarging spleen, and rapid intervention with transfusion have decreased morbidity and mortality associated with splenic sequestration.

Acute Chest Syndrome

This illness resembling pneumonia is the most common cause of death in children with sickle cell disease and the second most common cause of hospitalization.[19] Clinical findings include cough, fever, chest pain, dyspnea, chills, wheezing, and pulmonary infiltrates. Hemoglobin concentration and oxygen saturation decrease.[20] The etiology of acute chest syndrome is not clear. In children, an infectious agent can often be identified. Other possible causes include pulmonary edema from overhydration, fat embolism from infarcted bone marrow, and hypoventilation due to pain from rib infarcts or from narcotic analgesics used to combat pain. The long-term effects of recurrent episodes of acute chest syndrome are unknown.

Iron Overload

Excessive iron storage (hemosiderosis) is observed as a complication for some patients due to the use of transfusion therapy, erythropoiesis-induced increase in iron absorption, and an overall increase in longevity for patients with SCA. Hepatic fibrosis and cirrhosis related to hemosiderosis can occur. The serum ferritin assay is useful for monitoring storage iron levels in patients with SCA and can indicate the need for iron chelation therapy.

CASE STUDY (continued from page 235)

The chest radiograph showed consolidation in the left lower lobe, indicating that Shane has pneumonia.

5. What physiological conditions does Shane have that could lead to sickling of his erythrocytes?
6. What is the cause of Shane's pain and acute distress?
7. Why might Shane be more susceptible to pneumonia than an individual without sickle cell disease?

Laboratory Findings

Peripheral Blood

A normocytic, normochromic anemia is characteristic of sickle cell anemia; however, with marked reticulocytosis, the anemia can appear macrocytic (Figure 13-3 ■). Reticulocytosis from 10–20% is typical. The hemoglobin ranges from 6–10 g/dL (60–100 g/L) and the hematocrit from 18–30% (0.18–0.30 L/L). A calculated hematocrit from an electronic cell counter is more reliable than a centrifuged microhematocrit because excessive plasma trapped by sickled cells in centrifuged specimens falsely elevates the manual hematocrit.

The Cooperative Study of Sickle Cell Disease revealed that individuals homozygous for HbS have higher steady-state leukocyte counts than do normal individuals, especially children <10 years of age.[21] Platelet counts are also frequently higher than normal. After the age of 40, the hemoglobin concentration, reticulocyte count, leukocyte count, and platelet count decrease.[22]

The blood smear shows variable anisocytosis with polychromatophilic macrocytes and variable poikilocytosis with the presence of sickled cells and target cells. Nucleated erythrocytes can usually be found. The RDW is increased. During and following a hemolytic crisis, the RDW increases linearly with increases in reticulocytes.[23] If the patient is not experiencing a crisis, sickled cells may not be present.

In older children and adults, signs of splenic hypofunction are apparent on the peripheral blood smear with the presence of basophilic stippling, Howell-Jolly bodies, siderocytes, and poikilocytes.

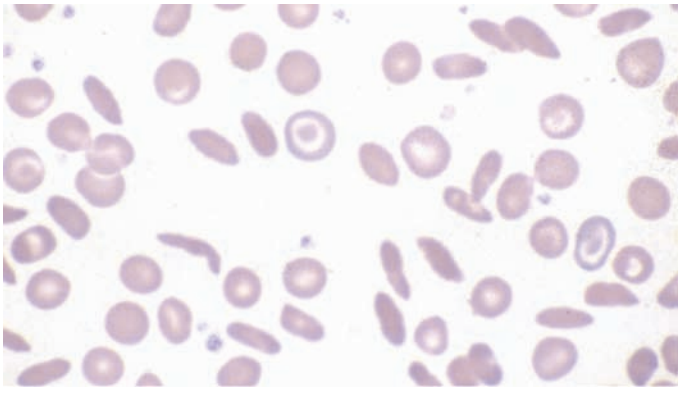
CASE STUDY

Admission laboratory data on Shane included:

WBC	16.4×10^9/L
RBC	2.5×10^{12}/L
Hb	78 g/L
Hct	0.24 L/L
PLT	467×10^9/L

Differential: Segs 76%, bands 10%, lymphs 9%, monos 3%, eos 1%, basos 1%; RBC morphology: Sickle cells 3+, target cells 1+, ovalocytes 1+, polychromasia, 3 NRBC/100 WBCs, Howell-Jolly bodies

8. Which of Shane's hematologic test results are consistent with a diagnosis of sickle cell anemia?
9. What does the presence of polychromatophilic erythrocytes signify?
10. Why is the absolute neutrophil count elevated?
11. What is the significance of ovalocytes on the blood smear?
12. What is the significance of Howell-Jolly bodies on the smear?



■ **FIGURE 13-3** Hemoglobin S disease (sickle cell anemia). Note abnormal boat-shaped, sickled, and ovoid erythrocytes (peripheral blood; Wright-Giemsa stain; 1000× magnification).

Bone Marrow

Bone marrow aspiration shows erythroid hyperplasia, reflecting the attempt of the bone marrow to compensate for chronic hemolysis. Erythrocyte production increases to 4–5 times normal. If the patient is deficient in folic acid, megaloblastosis can be seen. Iron stores are most often increased but can be diminished if hematuria is excessive. Bone marrow examination is not usually performed because it yields no definitive diagnostic information.

Hemoglobin Electrophoresis

The presence of HbS is often confirmed by hemoglobin electrophoresis, although other methods for its detection can be used. Electrophoresis on cellulose acetate at pH 8.4 shows 80–95% HbS (Table 13-3 ★). HbF ranges from 5–20%. High levels of HbF (25–35%) can indicate compound heterozygosity for HbS and hereditary persistence of fetal hemoglobin (Chapter 14). HbA_2 is normal. Newborns have 60–80% HbF with the remainder HbS. In infants <3 months of age with small amounts of HbS, electrophoresis on citrate agar gel at pH 6.2 permits more reliable separation of HbF from both HbA and HbS. Citrate agar gel electrophoresis is also useful in separating HbD and HbG from HbS. Both of these nonsickling hemoglobins migrate with HbS on alkaline electrophoresis with paper or starch gel but migrate with HbA on agar gel electrophoresis at acid pH.

Solubility Test

The solubility test is a rapid test for detecting HbS in the heterozygous or homozygous state. In severe anemia, the amount of HbS can be too low to be accurately detected, and the procedure may need to be altered. This test should not be used as a screening test for newborns because of high levels of HbF and the low concentration of HbS in this age group. Unstable hemoglobins can give a false positive test if many Heinz bodies are present. Other rare hemoglobin variants (e.g., HbC Harlem, HbI) can also give positive tests. False positive tests can occur with elevated plasma proteins and lipids.

Sickling Test

Another confirmatory test that is performed less often is the sodium metabisulfite slide test for sickling. This test is positive in both sickle cell anemia and sickle cell trait.

★ **TABLE 13-3** Hemoglobin Electrophoresis Results in Clinical Conditions

	Hemoglobin (%)				
Condition	**A**	**A2**	**F**	**S**	**C**
Normal adult	97	3	<1	0	0
Normal neonate	20–25	<1	75–80	0	0
*SCD (SS)	0	N	5–20	80–95	0
S trait (SA)	50–65	N	N	35–45	0
C disease	0	N	<7	0	>90
C trait (CA)	60–70	N	N	0	30–40
SC disease	0	?	?	>50	<50

*SCD = sickle cell disease

Other Diagnostic Tests

IEF in agar gels, CE, and HPLC can be used to identify abnormal hemoglobins such as HbS. Preferred methods for prenatal screening and diagnosis use DNA-based analysis (polymerase chain reaction) to detect point mutations in globin gene sequences. DNA testing provides a genotype diagnosis and eliminates the need for later neonatal testing when the phenotype results can be inconclusive. The molecular techniques are discussed in Chapter 42.

Other Laboratory Findings

Other laboratory findings are less specific. The hemolytic nature of the disease causes indirect bilirubin to increase, haptoglobin to decrease, and uric acid and serum lactic dehydrogenase (LD) to increase. Serum ferritin is typically increased. These tests offer no diagnostic information on sickle cell anemia but can be performed to evaluate complicating conditions.

CASE STUDY *(continued from page 239)*

The LD level was reported as 1260 U/L (reference interval 75–200 U/L).

13. What is the significance of Shane's elevated LD?

Therapy

Disease management and treatment for patients with SCA is extensive and multifaceted, and frequent monitoring of patient status using laboratory assays is needed. Treatment includes both immediate and prophylactic or long-term approaches. Immediate transfusion therapy may be ordered for complications of SCA, including stroke, vaso-occlusion in other organs, splenic sequestration, acute chest syndrome, aplastic crises, and others. Transfusion with units from normal donors dilutes the amount of HbS present, restores oxygenation of tissues, and temporarily suppresses erythropoiesis of new cells containing HbS. Simple transfusion or exchange transfusion may be used, often with units that are screened for HbS and fully (or partially) antigen matched.[24] Preoperative transfusion is helpful in preventing the complications of anesthesia-induced sickling. Long-term transfusion therapy can be useful in preventing complications of sickle cell anemia but is somewhat controversial due to potential side effects and lack of definitive treatment guidelines. Recent data have shown that transfusion therapy is useful in prevention of stroke and other complications in children with SCA.[25] Complications of chronic transfusion therapy include transmission of blood-borne diseases, hyperviscosity, alloimmunization, expense, inconvenience, and iron overload.

Various pharmacologic agents have been used to reduce intracellular sickling by increasing the level of HbF. Of these, only hydroxyurea (HU) has FDA approval for preventing vaso-occlusive crises in patients with sickle cell anemia. HU is widely used and elevates HbF in most HbS-containing erythrocytes by activating

genes controlling γ-globin chain production. Children and adults respond well to HU, experiencing fewer vaso-occlusive crises and hospitalizations. HU is an antineoplastic and antimetabolite drug with significant side effects of neutropenia, reticulocytopenia, and thrombocytopenia. Monitoring cell counts biweekly is recommended. HU is also a potential teratogen, so contraceptive precautions are recommended. HU does not seem to produce serious irreversible toxicity and is not associated with long-term adverse effects on growth or development in children.[26] Long-term risks of malignancy are unknown. Other pharmacologic agents for treating SCA are being investigated.

Additional aspects of treatment and patient management of SCA include prophylactic penicillin, folic acid, immunizations, hydration, and analgesics for pain.

Hematopoietic stem cell transplantation (SCT) affords the potential for the cure of sickle cell disease. Results of multicenter case series show disease-free survival rates ranging from 80–85% with the best outcomes obtained when children received stem cells from HLA-identical siblings. Risk of complications, including graft versus host disease and neurological problems, is high for patients with sickle cell disease undergoing SCT, especially when nonmyeloablative approaches or cells from unrelated donors are used.[27] Candidates for SCT include children with severe complications of SCA who have matched sibling donors. Furthermore, SCT is extremely expensive and can be beyond the financial means of many patients. Gene therapy, in which normal genes are inserted into a patient's defective stem cells and returned to the patient, also holds a promise of cure but is not available at this time. Progress in finding a stable gene vector capable of expressing therapeutic levels of normal β-globin chains over time has been slow.[28,29]

☑ CHECKPOINT 13-5

Outline the treatment options for a patient with HbS disease, pneumonia, and vaso-occlusive crisis. Discuss how each would affect the patient's clinical condition.

Sickle Cell Trait

Sickle cell trait is the heterozygous β^S state, and typically the patient has one normal β-gene and one β^S-gene ($\beta^A\beta^S$). Sickle cell trait is not as severe a disorder as sickle cell anemia because the presence of HbA or other non-S hemoglobins interferes with the process of HbS polymerization, preventing sickling under most physiologic conditions. However, sickle cell trait cells can sickle under very low oxygen tension (~15 mmHg). Although some hemoglobins other than HbS (especially HbC, HbD, HbE) can be incorporated into the polymer of deoxyhemoglobin S, the presence of molecules of these hemoglobins intermixed with the molecules of HbS creates a weakened structure and decreases the degree of polymerization. Because HbA constitutes >50% of the total hemoglobin, sickle cell trait rarely results in clinical symptoms and physical examinations are normal. However, it is important to diagnose sickle cell trait because, statistically, one of four children born to parents each of whom has the trait will have sickle cell anemia and two of four will have the trait.

Complications of splenic infarction and renal papillary necrosis have occasionally been reported in affected individuals subjected to extreme and prolonged hypoxia such as flying at high altitude in unpressurized aircraft or following general anesthesia.

Hematologic parameters, including hemoglobin concentration, are normal. No sickled cells or other abnormalities are observed on smears. Sickling can be induced with the sodium metabisulfite test, however, and the solubility test is also positive. Hemoglobin electrophoresis results show 50–65% HbA, 35–45% HbS, normal HbF, and normal or slightly increased HbA_2. If HbA constitutes <50% of the total hemoglobin in sickle cell trait, the patient is probably heterozygous for another hemoglobinopathy such as thalassemia.

OTHER SICKLING DISORDERS

Sickle cell disease/sickle cell disorder (SCD) are terms used to describe clinical conditions in which erythrocyte sickling occurs due to the presence of HbS. Compound heterozygous conditions (genotypes with two different hemoglobin mutations, heterozygous for each) may be considered SCD with variable clinical consequences. Some but not all of the clinical problems typical of sickle cell anemia apply to other SCD. Hydroxyurea and transfusion therapies could be needed, depending on disease severity. The combination of β^S with some forms of β thalassemia can be quite severe, requiring patient management and therapies comparable to those of sickle cell anemia. Hemoglobin electrophoresis and other methods including IEF and HPLC are needed to confirm the diagnosis of a combined disorder involving HbS. See "Hemoglobin S/C Disease" and Chapter 14 for additional information on combined disorders.

☑ CHECKPOINT 13-6

A child's parents both have sickle cell trait. The physician orders a hemoglobin electrophoresis on the child. Results of electrophoresis on cellulose acetate at pH 8.4 show 65% HbS, 30% HbA, 3% HbF, 2% HbA_2. Explain these results, and suggest further testing that could help in diagnosis.

HEMOGLOBIN C DISEASE

Hemoglobin C, the second hemoglobinopathy to be recognized, is the third most prevalent hemoglobin variant worldwide.[11] The first cases of HbC were discovered in the combined heterozygous state with HbS; this is not surprising because both hemoglobinopathies are prevalent in the same geographic area. Hemoglobin C is found predominantly in West African blacks in whom the incidence of the trait can reach 17–28% of the population. From 2–3% of African Americans carry the trait, and 0.02% have the disease.

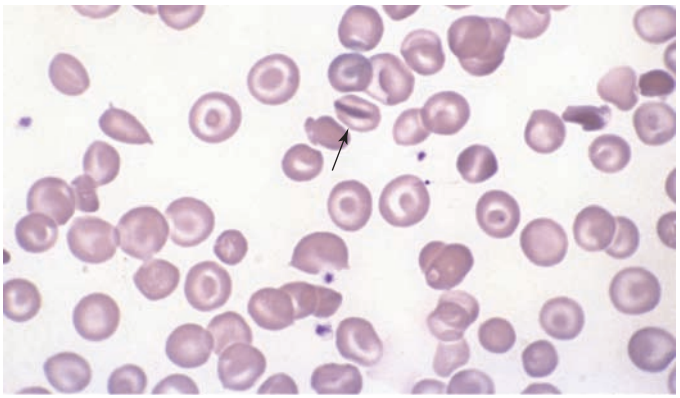


FIGURE 13-4 Hemoglobin C disease. Note the cell in the top center with a HbC crystal and target cells (peripheral blood; Wright-Giemsa stain; 1000× magnification).

Hemoglobin C is produced when lysine is substituted for glutamic acid at the sixth position (A3) in the β-chain [β6 (A3) Glu → Lys]. The mutation in the *HBB* gene is in the same position as the HbS mutation. Because the substitution is a nonpolar amino acid for a polar amino acid as with HbS, hemoglobin solubility decreases. Intraerythrocytic crystals of oxygenated HbC can be found in the red cells, especially in splenectomized individuals. HbF inhibits the formation of crystals. Crystal formation is enhanced when cells are dehydrated or in hypertonic solutions. Erythrocytes with crystals become rigid and are trapped and destroyed in the spleen, thereby reducing erythrocyte life span to 30–55 days.

Hemoglobin C disease ($\beta^C\beta^C$) is usually asymptomatic, but patients occasionally experience joint and abdominal pain. In contrast to sickle cell anemia, the spleen is most often enlarged. Variable hemolysis results in a mild to moderate anemia.

The hemoglobin ranges from 8–10 g/dL (80–120 g/L) and the hematocrit from 25–35% (0.25–0.35 L/L). The anemia is accompanied by a slight to moderate increase in reticulocytes. The stained blood smear contains small cells that appear to be folded and irregularly contracted as well as many target cells (Figure 13-4 ■). Intracellular hemoglobin crystals can be found if the smear has been dried slowly. Microspherocytes are occasionally present.

On cellulose acetate at an alkaline pH, HbC migrates with HbA_2, HbE, and HbO-Arab. HbC can be separated from these other hemoglobins by agar gel electrophoresis at an acid pH (Figure 13-1). For individuals with HbC disease, electrophoresis on citrate agar gel at acid pH demonstrates >90% HbC with a slight increase in HbF (not >7%).

Hemoglobin C trait (β^C/β^A) is asymptomatic. No hematologic abnormalities are produced except that target cells are noted on blood smears. Mild hypochromia can be present. About 60–70% of the hemoglobin is HbA and 30–40% is HbC.

HEMOGLOBIN S/C DISEASE

In hemoglobin S/C disease, both β-chains are structurally abnormal. One β-gene codes for β^S-chains, and the other gene codes for β^C-chains ($\beta^S\beta^C$); thus, HbA is absent. The combined heterozygous state for HbS and HbC results in a disease less severe then homozygous HbS but more severe than homozygous HbC. Hb S/C disease is considered a sickling disorder. The concentration of hemoglobin in individual erythrocytes (MCHC) is increased, and the concentration of HbS is more than in sickle cell trait (the percentage of HbS is higher than that of HbC because the β^C-globin has a less negative charge than the β^S-globin; thus, the cell does not form $\alpha\beta^C$-dimers as readily as $\alpha\beta^S$-dimers). The presence of HbC makes the HbS/C cells more prone to sickling than cells that contain HbA/HbS because HbC molecules participate in the polymerization process with HbS molecules more easily than do HbA molecules. Increased erythrocyte rigidity is noted at oxygen tensions <50 mm Hg. In addition, cells containing HbS/C have formation of aggregates of intracellular HbC crystals.[30] Thus, in HbS/C disease, both sickling and crystal formation contribute to the pathophysiology of the disease.

The clinical signs and symptoms of the disease are similar to those of mild sickle cell anemia, and treatment can be indicated. Because of the poorly deformable red cells, patients can develop vaso-occlusive crises leading to the complications associated with this pathology. A notable difference from sickle cell anemia, however, is that in HbS/C disease, splenomegaly is prominent.

Mild to moderate normocytic, normochromic anemia is present. The hematocrit is usually >25% (0.25 L/L), and the hemoglobin concentration is between 10 and 14 g/dL (100 and 140 g/L). The higher hemoglobin concentration does not necessarily mean that hemolysis is less severe than in sickle cell anemia; it could be that the higher oxygen affinity of HbS/C cells results in higher erythropoietin levels. Peripheral blood smears reveal a large number of target cells (up to 85%), folded cells, and boat-shaped cells but rarely sickled forms (Figure 13-5 ■). Typical HbC crystals are rarely found. Some erythrocytes contain a single eccentrically located, densely stained, round mass of hemoglobin that makes part of the cell appear empty. These cells have been referred to as *billiard-ball cells*.[30] Anisocytosis and poikilocytosis range from mild to severe. Small, dense, misshapen cells, some with crystals of various shapes jutting out at angles, have been referred to as *HbSC poikilocytes*.[31] Hemoglobin electrophoresis shows a higher concentration of HbS

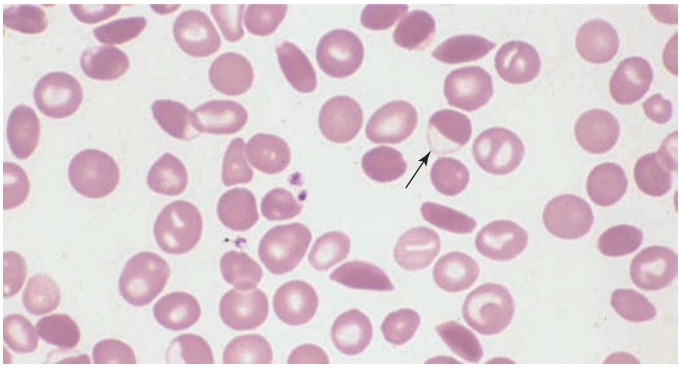


FIGURE 13-5 Hemoglobin S/C disease. Notice the elongated cells, the cell with hemoglobin contracted to one side of the cell (billiard ball cell; arrow), and boat-shaped cells. The small contracted cells are typical of those seen in hemoglobin C disease (peripheral blood; Wright-Giemsa stain; 1000× magnification).

than HbC. HbF can be increased up to 7%. No HbA is found due to the absence of normal β-chains.

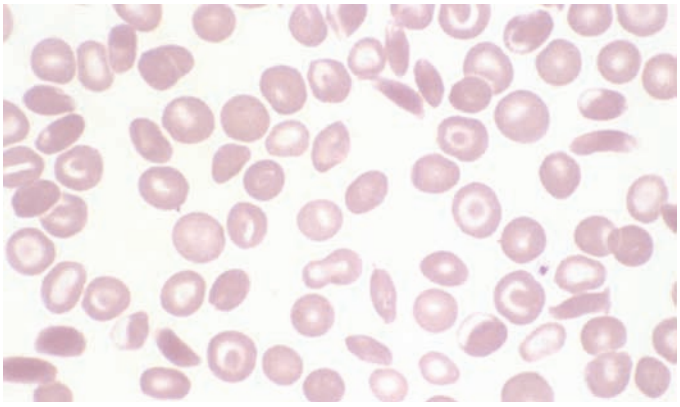
☑ CHECKPOINT 13-7

What is the functional abnormality of HbC and HbS? Why do these two abnormal hemoglobins have the same altered functions?

HEMOGLOBIN D

Hemoglobin D (HbD) is the result of several molecular variants of which the identical variants HbD Punjab and HbD Los Angeles (β121[GH4] Glu ⟶ Gln) are the most common. HbD migrates with HbS on hemoglobin electrophoresis at alkaline pH; however, the HbD molecules do not sickle and have normal solubility properties. HbD variants are found in various ethnic groups including Indians and African Americans. An uncommon α-chain variant, HbG Philadelphia, is also included in this group due to its similar electrophoretic properties. The rarely observed HbD homozygous state is associated with a mild hemolytic anemia and the presence of target cells, but the heterozygous state is asymptomatic. In homozygous HbD, electrophoresis on cellulose acetate at pH 8.4 demonstrates about 95% HbD with the same electrophoretic mobility as HbS. Electrophoresis on citrate agar at pH 6.0 allows separation of HbS and HbD. At acid pH, HbD migrates with HbA.

Although rare, the combined heterozygous state of HbD and HbS exists. HbD molecules can interact with HbS molecules, producing aggregates of deoxyhemoglobin (Figure 13-6 ■). This produces a relatively mild form of sickle cell anemia. HbD is also found in combination with β-thalassemia, resulting in a more serious clinical condition than when HbD is combined with a normal β allele. Compound heterozygotes can be recognized by results of electrophoresis and erythrocyte indices.



■ **FIGURE 13-6** A blood film from a patient with hemoglobin D/S. Homozygous HbD does not usually cause anemia, but when combined with HbS, it potentiates the aggregating of deoxyhemoglobin and sickling of erythrocytes, producing a mild sickle cell anemia. Notice the boat-shaped cells and target cells (peripheral blood; Wright-Giemsa stain; 1000× magnification).

☑ CHECKPOINT 13-8

A 13-year-old black female had a routine physical. Her CBC was normal, but the differential revealed many target cells. Hemoglobin electrophoresis revealed a band that migrated like HbS on cellulose acetate at pH 8.4. Her hemoglobin solubility test was negative. Explain the results and suggest a follow-up test to determine a diagnosis.

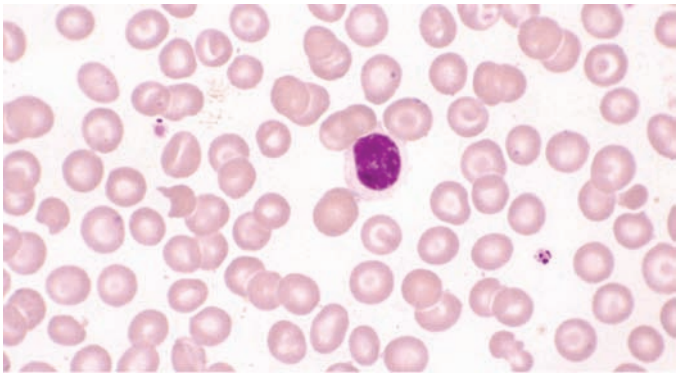
HEMOGLOBIN E

Hemoglobin E is the second most prevalent hemoglobinopathy worldwide.[11] It is most often encountered in individuals from southeast Asia. The trait has reached frequencies of almost 50% in areas of Thailand. It is estimated that 15–30% of immigrants from southeast Asia living in North America have HbE with the highest frequencies occurring in those from Cambodia and Laos. Although found mainly in Asians, the HbE trait can also occur in blacks.

Hemoglobin E is the result of a substitution of lysine for glutamic acid in the β-chain (β26[B8] Glu ⟶ Lys). The hemoglobin is slightly unstable when subjected to oxidant stress. Also, the nucleotide substitution creates a potential new splicing sequence so that some of the mRNA may be improperly processed. As a result, synthesis of the abnormal β-chain is decreased, and HbE trait and disease have some thalassemia-like characteristics including an increased ratio of α:non-α-chain synthesis and α-chain excess (Chapter 14). The oxygen dissociation curve is shifted to the right, indicating that HbE has decreased oxygen affinity.

Homozygous HbE is characterized by the presence of a mild, asymptomatic, microcytic hypochromic anemia (similar to thalassemia) with decreased erythrocyte survival (Figure 13-7 ■). Target cells are prominently observed on smears. Electrophoresis demonstrates mostly HbE (90% or more) with the remainder HbA_2 and HbF. On alkaline electrophoresis, HbE migrates with HbA_2, HbC, and HbO-Arab. On agar gel at an acid pH, HbE migrates with HbA.

HbE trait is asymptomatic, and hematologic parameters are normal except for slight microcytosis. Hemoglobin electrophoresis at



■ **FIGURE 13-7** A blood film from a patient with homozygous hemoglobin E (HbEE). Note the microcytosis (compare red cell size to that of the nucleus of the lymphocyte) and prominent target cells (peripheral blood; Wright-Giemsa stain; 1000× magnification).

alkaline pH shows about 35–45% HbE. The remainder is HbA with normal HbA_2 and HbF.

Combined heterozygosity for HbE and forms of thalassemia are commonly observed in some population groups. The degree of clinical severity is variable and depends on specific genotype. See Chapter 14.

☑ CHECKPOINT 13-9

The red cell morphology in HbE disease and β-thalassemia are similar: microcytic, hypochromic anemia with target cells. What laboratory test(s) could differentiate these two conditions?

UNSTABLE HEMOGLOBIN VARIANTS

Unstable hemoglobins can result from structurally abnormal globin chains, and >140 unstable variants have been described. The abnormal chains contain amino acid mutations at critical internal portions of the chains, which affect the molecular stability.[32] The disorders are characterized by denaturation and precipitation of the abnormal hemoglobin in the form of Heinz bodies, causing cell rigidity, membrane damage, and subsequent erythrocyte hemolysis. Although hemoglobin denaturation and hemolysis can occur spontaneously, symptoms associated with acute hemolysis usually occur after drug administration, infection, or other events that change the hemoglobin molecule's normal environment. Clinical conditions associated with unstable variants are known as *unstable hemoglobin disorders* or *congenital Heinz body hemolytic anemias*.

Pathophysiology

Most affected individuals with unstable hemoglobins are heterozygous. Most unstable hemoglobins are inherited as autosomal-dominant disorders, but a large number arise from spontaneous mutations with no evidence of hemoglobin instability in parents or other family members.

Unstable hemoglobin variants can result from a variety of globin chain amino acid substitutions or deletions that disrupt the stability of the globin subunit or hemoglobin tetramer, or the binding of heme to globin. Such mutations resulting in unstable hemoglobin variants include:

1. Mutation of a globin amino acid that is involved in contact with the heme group or that results in a tendency to dissociate heme from the abnormal globin chains
2. Replacement of nonpolar by polar amino acids at the interior of the molecule resulting in a distortion of the folding of the globin chain
3. Deletion or insertion of additional amino acids, particularly in the helical regions of the molecule, creating instability
4. Mutations of amino acids at intersubunit contacts (especially the $\alpha_1\beta_1$ contact points), creating instability and a tendency to dissociate into monomers
5. Replacement of a hydrophobic residue with a more hydrophilic amino acid in the hydrophobic pocket, disrupting the conformation of the hydrophobic heme cleft

Unstable hemoglobin denatures and precipitates as Heinz bodies, which attach to the inner surface of the membrane, thereby decreasing cell deformability. The inclusions are pitted by macrophages in the spleen, leaving the cell with less hemoglobin and decreased membrane. This process leads to rigid cells and their premature destruction in the spleen.

In addition to altering the molecule's stability, disruption of the normal conformation also can affect the molecule's function. Many of the unstable hemoglobins also have altered oxygen affinity. If the amino acid substitution is strategically located to affect the oxygen-binding site, oxygen affinity can be increased or decreased. In addition, some unstable hemoglobin variants have a tendency to spontaneously oxidize to methemoglobin.

Clinical Findings

Congenital hemolytic anemia indicative of an unstable hemoglobin disorder requires further investigation to establish a definitive diagnosis. Family history is extremely important in defining the hereditary nature of the disease. Patient history can also provide information about the nature of events such as infection or drug administration that precipitate acute hemolytic episodes. The severity of the disorder can range from asymptomatic to a chronic severe hemolytic anemia.

The severity of the anemia depends on the degree of instability of the hemoglobin tetramer and the change, if any, in oxygen affinity. For example, Hb Köln is an unstable hemoglobin (β-chain mutation) that also demonstrates increased oxygen affinity. Hemoglobins with increased oxygen affinity (hemoglobin-oxygen dissociation curve is shifted left) release less oxygen to the tissues, which results in relatively higher levels of tissue hypoxia and of erythropoietin as well as higher hematocrits than expected relative to the severity of hemolysis. Conversely, unstable hemoglobins with decreased oxygen affinity (e.g., Hb Hammersmith) have a hemoglobin-oxygen dissociation curve that is shifted right, increasing the oxygen delivery to the tissues and allowing patients to function at a lower hemoglobin concentration. When the oxygen affinity of an unstable hemoglobin is decreased, the reticulocyte count is not increased as much as would be expected for the degree of anemia relative to an uncomplicated hemolytic anemia.

Most clinical findings occur as the result of increased erythrocyte hemolysis. Jaundice and splenomegaly are common when there is chronic extravascular hemolysis. Cyanosis can result from the formation of sulfhemoglobin and methemoglobin that accompanies hemoglobin denaturation. Weakness and jaundice frequently follow the administration of oxidant drugs, which increase the hemoglobin's instability. Acute hemolysis can also be accompanied by the excretion of dark urine due to the presence of dipyrroles in the urine.

Laboratory Findings

Peripheral Blood

The anemia of congenital hemolytic anemia involving unstable hemoglobin variants is usually normocytic and normochromic. Occasionally, MCV and MCH are slightly decreased because of the removal of Heinz bodies from erythrocytes and loss of hemoglobin in the spleen.

The reticulocyte count is typically increased. The blood smear can show basophilic stippling, pitted cells (bite cells), and small contracted cells.

If splenic function is efficient, Heinz bodies are not detectable in peripheral blood cells. Heinz bodies can be found in the peripheral blood following splenectomy; however, this finding is not specific for unstable hemoglobin disorders. Heinz bodies can also be seen in erythrocyte enzyme abnormalities that permit oxidation of hemoglobin, in thalassemia, and after administration of oxidant drugs in normal individuals. Heinz bodies of unstable hemoglobins can be generated in vitro by incubation of the erythrocytes with brilliant cresyl blue or other redox agents. These intracellular inclusions cannot be observed on smears stained with Wright's stain.

Other Laboratory Findings

Many unstable hemoglobins identified to date have the same charge and electrophoretic mobility as normal hemoglobin. Only about 45% can be identified by electrophoresis. Hemoglobin A_2 and HbF are sometimes increased, which could suggest the presence of an abnormal hemoglobin when it is not detected by its electrophoretic pattern.

The presence of an unstable hemoglobin (i.e., one that precipitates more readily than normal hemoglobin) may be demonstrated by screening tests, including the heat denaturation test or isopropanol precipitation test. When a hemoglobin solution is heated to 50°C for 1 hour, an unstable hemoglobin variant will show precipitation. Normal hemoglobins will not precipitate. An unstable hemoglobin is also precipitated within 20 minutes in the isopropanol (17% by volume) precipitation test. Normal hemoglobin remains in solution 30–40 minutes. More definitive identification of unstable hemoglobin variants can be made by using isoelectric focusing, HPLC analysis of globin chains, or genetic testing for globin gene abnormalities.

☑ CHECKPOINT 13-10

a. Explain why patients with an unstable hemoglobin variant usually experience acute hemolysis only after administration of certain drugs or with infections.

b. A patient is suspected of having a congenital Heinz body hemolytic anemia but hemoglobin electrophoresis is normal. Why is it necessary to perform additional tests?

Therapy

A wide variation in the clinical presentation of patients with unstable hemoglobin variants exists, and many do not require therapy. Splenectomy can be performed if hemolysis is severe. Patients are advised to avoid oxidizing drugs, which can precipitate a hemolytic episode.

HEMOGLOBIN VARIANTS WITH ALTERED OXYGEN AFFINITY

Amino acid substitutions in the globin chains near the heme pocket can affect the hemoglobin's ability to carry oxygen by preventing heme from binding to the globin chain or by stabilizing iron in the oxidized ferric state. Other substitutions that affect oxygen affinity include those near the $\alpha_1\beta_2$ contacts, at the C terminal end of the β-chain, and near the 2,3-BPG binding site. These are critical sites involved in the allosteric properties of hemoglobin and/or in the physiologic regulation of hemoglobin affinity for oxygen. Either the α- or β-chain can be affected, but most identified substitutions are associated with the β-chain.

Increased oxygen affinity results in congenital erythrocytosis, a compensatory mechanism for the reduced release of oxygen to the tissues. Hemoglobinopathies causing permanent methemoglobin formation are associated with pseudocyanosis, whereas cyanosis characterizes hemoglobins with decreased oxygen affinity.

Hemoglobin Variants with Increased Oxygen Affinity

High-affinity hemoglobins are inherited as autosomal dominant traits. All such hemoglobins discovered have been in the heterozygous state. The variants can result from amino acid substitutions that involve the $\alpha_1\beta_2$ contacts. Mutations alter the quaternary structure of hemoglobin to favor the R (oxy) state by either stabilizing this conformation or destabilizing the T (deoxy) state. Other substitutions affect the C-terminal end of the β-chain, which is important in maintaining the stability of the T form. A few substitutions affect the 2,3-BPG binding sites, which alter oxygen affinity when bound to hemoglobin.

The high-affinity hemoglobins bind oxygen more readily than normal and retain more oxygen at lower PO_2 levels, which results in a shift to the left of the oxygen dissociation curve. The P_{50} of the hemoglobin is decreased to 12–18 mm Hg, meaning that less oxygen is released to tissues at the tissue PO_2 of ~26 mm Hg. The resulting tissue hypoxia stimulates erythropoietin release and, subsequently, formation of a compensatory increased erythrocyte mass. In addition to the primary effect of increasing oxygen affinity, secondary effects of the mutations, including hemoglobin instability, reduced Bohr effect, and reduced cooperativity of the oxygen binding, can also occur.[32]

Erythrocyte counts and hematocrit levels are increased, and hemoglobin levels are increased to about 20 g/dL (200 g/L). Other hematologic parameters are normal. About half of the hemoglobin variants have an altered electrophoretic mobility, enabling diagnosis by starch gel or cellulose acetate electrophoresis. Diagnosis is established by measuring oxygen affinity (determining the P_{50} of the patient's hemoglobin).

Individuals with these hemoglobin variants are asymptomatic. A ruddy complexion is occasionally apparent as a result of the erythrocytosis. The importance of identifying the presence of a high-affinity hemoglobin is for the differential diagnosis and exclusion of other causes of erythrocytosis and the avoidance of unnecessary and expensive diagnostic and therapeutic interventions.

Hemoglobin Variants with Decreased Oxygen Affinity

Low oxygen-affinity hemoglobins result from mutations that stabilize or favor the deoxygenated (T) conformation of the hemoglobin molecule or destabilize the oxygenated (R) form. These mutations impair oxygen binding or reduce heme–heme subunit interactions (cooperativity). Low oxygen-affinity hemoglobins are often associated with mutations involving the $\alpha_1\beta_2$ contact points, which are involved in

intramolecular movement as hemoglobin goes from the oxy (R) to the deoxy (T) state. The low-affinity variants result in a right-shifted oxygen dissociation curve.

Most low oxygen-affinity hemoglobins possess enough oxygen affinity to become fully saturated in the lungs but at the capillary PO_2 in the peripheral tissues, they deliver higher than normal amounts of oxygen (i.e., become more unsaturated). Two physiologic effects result:

- Because oxygen delivery is so efficient, oxygen requirements of the tissues can be met by lower than normal hemoglobin concentration, producing a pseudoanemia (hemoglobin lower than "normal").
- The amount of deoxygenated hemoglobin in the capillaries and veins is increased, resulting in cyanosis. However, no adverse clinical effect is associated with this cyanosis. Patients are asymptomatic and require no treatment.

Diagnosis requires determining the P_{50} (the PO_2 at which hemoglobin is 50% saturated). HPLC can also be useful in identifying hemoglobin variants with low oxygen affinity.

Methemoglobinemias

Methemoglobin is hemoglobin with iron oxidized to the ferric state, which cannot carry oxygen. **Methemoglobinemia** is a clinical condition that occurs when methemoglobin encompasses >1% of the hemoglobin (Chapter 6). This condition can occur as a result of either acquired or inherited defects in the methemoglobin reductase system or as a result of the presence of a structurally abnormal globin chain. In the latter case, the defect results in increased formation of methemoglobin by rendering the molecule relatively resistant to reduction by methemoglobin reductase.

This structural variant of hemoglobin is called *hemoglobin M* (*HbM*). Nine variants of it have been described; all variants have been found only in the heterozygous state. Most HbM variants are produced by a tyrosine substitution for the proximal or distal histidine in the heme pocket of the α- or β-chains. Tyrosine forms a covalent link with heme iron, stabilizing the iron in the ferric state. The stabilized, oxidized iron is relatively resistant to reduction by the methemoglobin reductase pathway. If the substitution occurs in the α-chain, cyanosis (or actually pseudocyanosis) is present from birth because the α-chain is a component of HbF ($\alpha_2\gamma_2$), the major hemoglobin at birth. If the substitution occurs in the β-chain, cyanosis does not occur until about the sixth month after birth when HbA ($\alpha_2\beta_2$) becomes the major hemoglobin.

The presence of methemoglobin imparts a brownish color to the blood. Except for this abnormal color, no other hematologic abnormality is present because methemoglobin levels are rarely higher than 30%. Patients with methemoglobinemia appear to be cyanotic, but unlike truly cyanotic people, arterial blood PO_2 levels are usually normal (i.e., pseudocyanosis).

Hemoglobin M does not always separate from HbA at an alkaline pH. Hemoglobin electrophoresis on agar gel at pH 7.1 of a blood sample containing HbM reveals a brown band (HbM) running anodal to a red band (HbA). The pattern can appear sharper with IEF. Oxidizing the erythrocyte hemolysate with ferricyanide before electrophoresis reveals a sharp separation of congenital HbM and methemoglobin formed from HbA. Methemoglobin formed from HbA by oxidation of iron has no change in molecular charge and therefore has the same electrophoretic mobility as HbA.

Although HbM can be detected by spectral abnormalities of the hemoglobin (absorption peaks at ~630 and 502 nm), methemoglobin formed in NADH-diaphorase deficiency has a normal absorption spectrum. In addition, methemoglobin formed as the result of NADH-diaphorase deficiency can be readily reduced by incubation of blood with methylene blue whereas methemoglobin caused by a structural hemoglobin variant such as HbM does not reduce with the methylene blue. To confirm NADH-diaphorase deficiency, a quantitative assay of the enzyme activity is necessary. Refer to Table 13-4 ★ for a summary of differentiation of types of methemoglobinemia (see also Chapter 6, Table 6-6).

No treatment for the HbM structural variants exists because the abnormal hemoglobin resists reduction. However, most patients are asymptomatic and require no management. Most patients have methemoglobin levels below the level at which symptoms of oxygen deprivation occur.

☑ CHECKPOINT 13-11

Why should red cell enzyme assays and hemoglobin electrophoresis both be performed on a patient with congenital cyanosis?

★ **TABLE 13-4** Causes and Characteristics of Methemoglobinemia

Type	Cause	Electrophoretic Pattern	NADH-Diaphorase Activity	Reduction with Methylene Blue
Acquired	Hb oxidized at a rate exceeding capacity of methemoglobin reductase system	Normal	Normal	Yes
Congenital				
Recessive	Defect in methemoglobin reductase system	Normal	Decreased	Yes
Dominant	Structural abnormality of hemoglobin (HbM)	HbM variant	Normal	No

Summary

The hemoglobinopathies are a group of chronic hemolytic anemias caused by qualitative defects in the globin chains of hemoglobin. Quantitative defects in globin chain synthesis (thalassemias) are discussed in Chapter 14.

Qualitative defects are due to genetic mutations that cause a structural change in the globin chain. Although the mutation can affect any of the globin chain classes, most clinically significant mutations affect the β-chain. The mutation can affect the hemoglobin molecule's solubility, stability, or function (oxygen affinity). Hemoglobin electrophoresis can be used to detect those mutants in which amino acid mutations cause a change in the hemoglobin molecule's electrophoretic mobility. However, not all variants can be detected and identified by electrophoresis, and other methods such as HPLC, IEF, or CE can be used. Other tests detect alterations in hemoglobin solubility and/or stability or oxygen affinity. Molecular techniques are available to help definitively diagnose hemoglobinopathies but are not always necessary for routine diagnostic purposes.

In the United States, the most common structural variants are HbS and HbC. Both are characterized by decreased hemoglobin solubility and can be detected electrophoretically. Hemoglobin S has decreased solubility when deoxygenated, forming rigid aggregates and reduced erythrocyte deformability. The rigid cells aggregate in the microvasculature, resulting in tissue hypoxia. In HbC disease, the erythrocytes become trapped and destroyed in the spleen when intracellular crystals of HbC form. Generally, these variants produce a normocytic, normochromic anemia with anisocytosis and poikilocytosis characteristic for the specific hemoglobinopathy. The blood smear in HbS disease can show sickled forms and target cells. HbC disease has numerous target cells and irregularly contracted cells, and the erythrocytes occasionally contain HbC crystals. In the United States, both HbS and HbC are found in highest frequency among African Americans. Hemoglobin E is especially prevalent in people from southeast Asia. HbE disease is characterized by a mild, asymptomatic microcytic anemia.

Unstable hemoglobin variants produce *congenital Heinz body hemolytic anemia*. The hemoglobin denatures in the form of Heinz bodies, decreasing cell deformability and resulting in membrane damage and premature destruction. Hemoglobin variants with increased or decreased oxygen affinity can result in erythrocytosis or cyanosis, respectively. Hemoglobin variants resulting in methemoglobin formation are associated with pseudocyanosis.

Review Questions

Level I

1. A patient has been previously diagnosed as being heterozygous for hemoglobin D. What is an important identifying characteristic of this hemoglobin variant? (Objective 7)

   A. It migrates with HbS on hemoglobin electrophoresis at alkaline pH.

   B. Sickled RBCs are typically observed on smears.

   C. Hemoglobin electrophoresis at alkaline pH is needed for its separation from another common variant.

   D. It causes severe hemolytic anemia with many target cells in heterozygotes.

2. Which of the following laboratory assays are useful in recognizing and confirming a diagnosis for patients with hemoglobinopathies? (Objective 6)

   1. Hemoglobin electrophoresis
   2. RBC indices
   3. RBC morphology
   4. Hemoglobin level
   5. Chemistry assays including bilirubin, ferritin, and others

   A. 1 and 5 only

   B. 2 and 4 only

   C. 1, 3, and 4 only

   D. all of the above

3. Which part of the standard CBC provides the most useful screening information when differentiating between a patient diagnosis of sickle cell anemia versus thalassemia? (Objective 6)

   A. hemoglobin concentration alone

   B. hemoglobin and hematocrit together

   C. erythrocyte indices

   D. total WBC count and differential

4. Which homozygous hemoglobinopathy is most consistent with the following laboratory test results? (Objective 7)

   Hemoglobin level mildly decreased, MCV decreased, target cells and hypochromia observed on smear, a large band in the A_2 region on alkaline electrophoresis

   A. hemoglobin C

   B. hemoglobin D

   C. hemoglobin E

   D. hemoglobin S

5. Which of the following is a clinical condition whose associated laboratory findings are seen in children with sickle cell anemia? (Objectives 7, 8)

   A. vaso-occlusive crisis denoted by the presence of many sickled RBCs on smears

   B. erythrocyte aplasia denoted by a rapid increase in hemoglobin

   C. bacterial infection denoted by the appearance of Howell-Jolly bodies on smears

   D. thrombosis denoted by an increase in WBCs and abnormal differential

6. Hemoglobinopathies are clinical diseases that result from genetically determined abnormalities of the hemoglobin molecule and include those involving: (Objective 1)

   1. heme structure
   2. decreased heme synthesis
   3. globin chain structure
   4. reduced globin chain synthesis

   A. 1 and 3 only

   B. 1 and 2 only

   C. 3 and 4 only

   D. all of the above

7. Amino acid substitutions on globin chains often alter the hemoglobin molecule's charge and mobility. This is the principle of which test for identifying hemoglobins? (Objective 3)

   A. HbA_2 quantitation

   B. HbF quantitation

   C. hemoglobin electrophoresis

   D. solubility test

8. Tests that quantitate HbF and HbA_2 are useful in detecting hemoglobinopathies because: (Objective 6)

   A. they are the only valid tests available for this purpose

   B. they are routinely performed in most laboratories

   C. HbF and HbA_2 are typically decreased in hemoglobinopathies

   D. HbF and HbA_2 are typically increased in hemoglobinopathies

9. The presence of many sickled erythrocytes on a peripheral blood smear is most likely to be found in a person who is: (Objective 7)

   A. heterozygous for HbC

   B. homozygous for HbC

   C. heterozygous for HbS

   D. homozygous for HbS

10. A hemoglobin electrophoresis showed 49% HbS, 42% HbC, 6% HbF, and 3% HbA_2. These results are consistent with a diagnosis of: (Objective 7)

    A. sickle cell anemia

    B. sickle cell trait

    C. hemoglobin S/C disease

    D. hemoglobin C trait

Level II

1. In which U.S. population group would you expect to observe the lowest prevalence of clinically significant hemoglobinopathies? (Objective 2)

   A. African Americans

   B. Caucasian Americans

   C. Immigrants from southeast Asia

   D. Immigrants from the Mediterranean basin

2. An African American 2-year-old is seen in an outpatient clinic with signs and symptoms consistent with a severe hemoglobinopathy. Based on this presentation, this child is most likely homozygous for which hemoglobin variant? (Objective 5)

   A. HbC

   B. HbD

   C. HbE

   D. HbS

3. A patient with a long history of anemia is referred to a hematologist for diagnosis. This patient recently was treated with a drug for an unrelated condition, but the therapy was discontinued following an acute episode of severe anemia. Other family members also appear to suffer from a similar condition. The hematologist suspects the patient has an unstable hemoglobin variant and asks for test suggestions. What laboratory results would best support the preliminary diagnosis? (Objective 10)

   A. Heinz bodies observed upon staining, increased reticulocytes, bite cells on peripheral blood smear

   B. decreased reticulocytes, decreased indices, target cells on smears

   C. Howell-Jolly bodies and sickle cells observed on smears

   D. increased WBC concentration with abnormal differential

4. What is an advanced testing method suitable for detecting and identifying hemoglobin variants that you might select for your high-volume laboratory in a large, urban medical center? (Objective 9)
   A. hemoglobin electrophoresis performed at alkaline pH only
   B. hemoglobin solubility test
   C. high-performance liquid chromatography
   D. hemoglobin electrophoresis performed at acid pH only
5. An asymptomatic patient presents with persistent erythrocytosis. The hematologist has ruled out polycythemia vera and other causes. What is an additional explanation for the erythrocytosis, and what laboratory assay might be useful in this case? (Objective 11)
   A. an unstable hemoglobin variant identified by performing a Heinz body stain
   B. a high-affinity hemoglobin variant identified by isoelectric focusing
   C. a crystalizable hemoglobin variant demonstrated by observing hemoglobin C crystals on a smear
   D. a quantitative decrease in globin chain production indicated by observing an increased level of HbF
6. Rank the following hemoglobinopathies on the basis of quantity of HbS starting with the lowest amount. (Objective 1)
   1. adult with sickle cell disease
   2. adult with sickle cell trait
   3. neonate with sickle cell disease

   A. 3, 2, 1
   B. 2, 3, 1
   C. 1, 2, 3
   D. 2, 1, 3
7. HbS is an example of a hemoglobin with a globin chain mutation that alters hemoglobin: (Objective 3)
   A. function
   B. solubility
   C. stability
   D. oxygen binding
8. Cells containing large amounts of HbS sickle when which of the following conditions occur? (Objective 4)
   A. high oxygen tension and acidosis
   B. hypoxia and alkalosis
   C. temperatures $<37°C$ and alkalosis
   D. temperatures $>37°C$ and hypoxia
9. A hemoglobin electrophoresis on cellulose acetate at pH 8.5 is performed on a 2-year-old African American patient with severe anemia. An abnormal band appears halfway between the HbA and HbA_2 positions on the strip. The HbF level is 10%, and the HbA_2 level is normal. No HbA is present. What is the most likely identity of the abnormal hemoglobin? (Objective 7)
   A. HbC
   B. HbS
   C. HbD
   D. HbE
10. The presence of HbE disease in an adult is best confirmed using which routine laboratory test? (Objective 8)
    A. hemoglobin electrophoresis
    B. CBC and peripheral blood smear
    C. solubility test
    D. PCR for molecular defect

Companion Resources

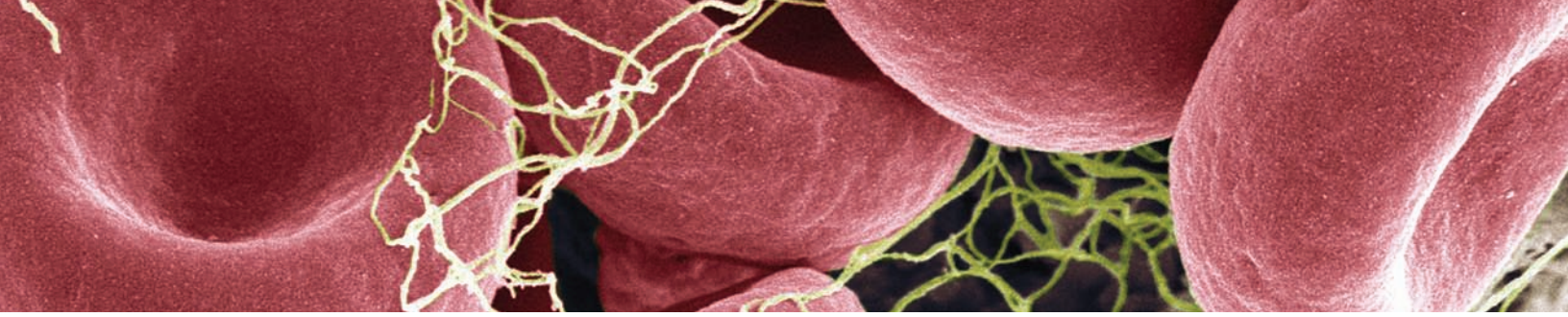
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Weatherall DJ, Clegg JB. Genetic disorders of hemoglobin. *Semin Hematol*. 1999;36(4)(suppl 7):24–37.
2. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. *Bull World Health Organ*. 2008;86:480–487.
3. Herrick JB. Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. *Trans Assoc Am Physicians*. 1910;25:553–61.
4. Pauling L, Itano HA, Singer SJ et al. Sickle cell anemia: A molecular disease. *Science*. 1949;110:543–48.
5. Huisman TH, Carver MF, Baysal E et al. A database of human hemoglobin variants and thalassemias. http://globin.cse.psu.edu/globin/hbvar/menu.html (accessed March, 1414).
6. Clarke GM, Higgins TN. Laboratory investigation of hemoglobinopathies and thalassemias: Review and update. *Clin Chem*. 2000;46:1284–90.
7. Abou-Diwan C, Young AN, Molinaro RJ. Hemoglobinopathies and clinical laboratory testing. *Med Lab Obs*. 2009 41:8–18.
8. Houtovsky A, Hadzi-Nesic J, Nardi MA. HPLC retention time as a diagnostic tool for hemoglobin variants and hemoglobinopathies: a study of 60,000 samples in a clinical diagnostics laboratory. *Clin Chem*. 2004:50:1736–47.
9. U.S. Preventive Services Task Force. Screening for sickle cell disease in newborns: U.S. Preventive Services Task Force recommendation statement. Rockville (MD): Agency for Healthcare Research and

Quality (AHRQ); 2007 (available at www.guideline.gov; accessed March 14, 2014).
10. Old JM. Screening and genetic diagnosis of haemoglobin disorder. *Blood Rev*. 2003;17:43–53.
11. Flint J, Harding RM, Boyce AJ et al. The population genetics of the haemoglobinopathies. *Baillieres Clin Haematol*. 1998;11:1–51.
12. Bunyaratvej A, Butthep P, Sae-Ung N et al. Reduced deformability of thalassemic erythrocytes and erythrocytes with abnormal hemoglobins and relation with susceptibility to *Plasmodium falciparum* invasion. *Blood*. 1992;79:2460–63.
13. Liu SC, Derick LH, Palek J. Dependence of the permanent deformation of red blood cell membranes on spectrin dimer-tetramer equilibrium: implication for permanent membrane deformation of irreversibly sickled cells. *Blood*. 1993;81:522–28.
14. Ballas SK, Smith ED. Red blood cell changes during the evolution of the sickle cell painful crisis. *Blood*. 1992;79:2154–63.
15. Mackie LH, Hochmuth RM. The influence of oxygen tension, temperature, and hemoglobin concentration on the rheologic properties of sickle erythrocytes. *Blood*. 1990;76:1256–61.
16. Steinberg MH. Pathophysiology of sickle cell disease. *Baillieres Clin Haematol*. 1998;11:163–84.
17. Liu SC, Yi SJ, Mehta JR et al. Red cell membrane remodeling in sickle cell anemia. *J Clin Invest*. 1996;97:29–36.
18. Boghossian SH, Nash G, Dormandy J et al. Abnormal neutrophil adhesion in sickle cell anaemia and crisis: relationship to blood rheology. *Br J Haematol*. 1991;78:437–41.
19. Ballas SK. Sickle cell disease: Clinical management. *Baillieres Clin Haematol*. 1998;11:185–14.
20. Quinn CT, Buchanan GR. The acute chest syndrome of sickle cell disease. *J Pediatr*. 1999;135:416–22.
21. West MS, Wethers D, Smith J et al. Laboratory profile of sickle cell disease: a cross-sectional analysis. The cooperative study of sickle cell disease. *J Clin Epidemiol*. 1992;45:893–909.
22. Morris J, Dunn D, Beckford M et al. The haematology of homozygous sickle cell disease after the age of 40 years. *Br J Haematol*. 1991;77:382–85.
23. el Sayed HL, Tawfik ZM. Red cell profile in normal and sickle cell diseased children. *J Egypt Soc Parasitol*. 1994;24:147–54.
24. Quirolo K. How do I transfuse patients with sickle cell disease? *Transfusion*. 2010;50:1881–86.
25. Miller ST, Wright E, Abboud M. Impact of chronic transfusion on incidence of pain and acute chest syndrome during the Stroke Prevention Trial (STOP) in sickle-cell anemia. *J Pediatr*. 2001;139:785–89.
26. Trompeter S, Roberts I. Haemoglobin F modulation in childhood sickle cell disease. *Br J Haematology*. 2008;144:308–16.
27. Thompson AA. Advances in the management of sickle cell disease. *Pediatr Blood Cancer*. 2005;46:533–39.
28. Persons DA, Nienhuis AW. Gene therapy for the hemoglobin disorders: past, present, and future. *Proc Natl Acad Sci USA*. 2000;97(10):5022–24.
29. Walters MC. Stem cell therapy for sickle cells disease: transplantation and gene therapy. *Hematology 2005*. Washington DC: American Society for Hematology. 2005:66–73
30. Lawrence C, Fabry ME, Nagel RL. The unique red cell heterogeneity of SC disease: crystal formation, dense reticulocytes, and unusual morphology. *Blood*. 1991;78:2104–12.
31. Bain BJ. Blood film features of sickle cell–haemoglobin C disease. *Br J Haematol*. 1993;83:516–18.
32. Steinberg MH. Hemoglobins with altered oxygen affinity, unstable hemoglobins, M-hemoglobins, and dyshemoglobinemias. In: Greer JP, Foester J, Rodgers GM, et al., eds. *Wintrobe's Clinical Hematology*, 12th ed. Philadelphia; Wolters Kluwer Health/Lippincott Williams & Wilkins; 2009.



14 Thalassemia

TIM R. RANDOLPH, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *thalassemia*.
2. Differentiate thalassemias from hemoglobinopathies based on definition and basic pathophysiology.
3. Describe the typical peripheral blood morphology associated with thalassemia.
4. Compare and contrast the etiology of α- and β-thalassemia.
5. For each of the four genotypes of α-thalassemia, describe the:
   a. Number of affected alleles
   b. Individuals affected
   c. Basic pathophysiology
   d. Symptoms
   e. Laboratory results including blood cell morphology and hemoglobin electrophoresis
6. For each of the six genotypes of β-thalassemia describe the:
   a. Individuals affected
   b. Basic pathophysiology
   c. Symptoms
   d. Laboratory results including blood cell morphology and hemoglobin electrophoresis

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. List and describe five primary genetic defects found in thalassemias.
2. Compare and contrast α- and β-thalassemia.
3. Correlate the outcomes in hemoglobin synthesis resulting from the five genetic defects in thalassemia.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

4. For all four genotypes of α-thalassemia:
   a. Correlate all three nomenclature systems: genotype, genotype description, and phenotype.
   b. Explain advanced pathophysiology.
   c. Describe treatment and prognosis.
5. For all four phenotypes of β-thalassemia:
   a. List expected genotypes.
   b. Explain advanced pathophysiology.
   c. Describe treatment and prognosis.
6. Correlate clinical severities of both α- and β-thalassemia with their respective genotypes.
7. Compare and contrast other thalassemia and thalassemia-like conditions including:
   a. $\delta\beta$-thalassemia
   b. $\gamma\delta\beta$-thalassemia
   c. Hemoglobin Constant Spring
   d. Hereditary persistence of fetal hemoglobin (HPFH)
   e. Hemoglobin Lepore
   f. Thalassemia/hemoglobinopathy combination disorders
8. Differentiate iron deficiency-anemia and HPFH from thalassemia based on results of laboratory tests and clinical findings.

Key Terms

Allele
Compression syndrome
Crossover
Diploid
Double heterozygous
Extramedullary erythropoiesis
Functional hyposplenism
Gene cluster
Genotype
Haplotype
Heterozygous
Homozygous
Ineffective erythropoiesis
P_{50} value
Phenotype
Zygosity

Background Basics

The information in this chapter builds on concepts presented in previous chapters. To maximize your learning experience, you should review the following concepts before beginning this unit of study:

Level I

- Describe the pathophysiology of hemoglobinopathies. (Chapter 13)
- Describe the morphologic and functional classification of anemias and the associated lab tests. (Chapter 11)
- Interpret routine laboratory tests, such as CBC and differential, and apply both normal and abnormal results in the diagnosis of anemias. (Chapters 10, 11, 37)
- Describe the basic structure and function of hemoglobin and identify the globin chain composition of normal hemoglobin types. (Chapter 6)
- Describe the extravascular destruction of erythrocytes and degradation of hemoglobin. (Chapters 5, 6)

Level II

- Summarize the synthesis and molecular structure of hemoglobin and correlate alterations in structure with function; describe globin chain synthesis in utero and throughout life. (Chapters 3, 6)
- Interpret hemoglobin migration patterns on electrophoresis, acid elution of fetal hemoglobins, hemoglobin solubility tests, and iron panels to distinguish iron metabolism disorders and hemoglobinopathies from thalassemias. (Chapters 12, 13, 37)

CASE STUDY

We will refer to this case throughout the chapter.

John is a 4-year-old boy who frequently complains of weakness, fatigue, and dyspnea. The family moved to the United States from Greece before the child's birth. Both parents experienced fatigue from time to time but never consulted a physician. Consider the types of anemia most often found at this age and the laboratory tests that could help establish a diagnosis.

What is the significance, if any, of knowing the parents' background and medical history?

OVERVIEW

This chapter discusses a group of hereditary anemias collectively called *thalassemia*. It begins with a general description of thalassemia, including the genetic defects and types, pathophysiology, and clinical and laboratory findings. Subsequently, the following are discussed for each type: pathophysiology, clinical findings, laboratory results, treatment, and prognosis. Other thalassemia-like conditions are described and compared and contrasted with thalassemia. The chapter concludes by describing the laboratory differential diagnosis of types of thalassemia and other disorders that have similar peripheral blood morphology.

INTRODUCTION

Thalassemia constitutes a family of inherited disorders in which mutations in one or more of the globin genes of hemoglobin cause decreased or absent synthesis of the corresponding globin chains. More than 400 unique mutations have been described among this diverse group of disorders.[1] Consequences of the mutation depend on the particular chain affected and the amount of globin chain produced. Limited availability of globin chains results in a reduction in the assembly of hemoglobin. Patients with mild genetic defects are generally asymptomatic. Patients with more severe

defects present with symptoms that result from one or more of the following: decreased production of normal hemoglobin, synthesis of abnormal hemoglobins, unbalanced synthesis of α- and non-α-globin chains and **ineffective erythropoiesis**. Symptoms include anemia, hepatosplenomegaly, infections, gallstones, and bone deformities that alter facial features and result in pathologic fractures.

The thalassemias are classified according to the affected globin chain. Clinically, the most important thalassemias involve the α- and β-chains because they are components of hemoglobin A, which makes up 97% of normal adult hemoglobin. α-Thalassemia results from decreased or absent production of α-globin chains, and β-thalassemia is caused by decreased or absent production of β-globin chains. Reduction in the synthesis of the δ-chain, a component of hemoglobin A_2, can occur but is not associated with anemia. Severe impairment of ζ-, ϵ-, or γ-globin synthesis is usually lethal in utero.

Thomas Cooley offered the first clinical description of thalassemia in Detroit in 1925.[2] At that time, thalassemia was thought to be a rare disorder restricted to the Mediterranean ethnicities. Dr. Cooley's work broadened our understanding of the nationalities that potentially could be affected by thalassemia and suggested that the disease was hemolytic in nature. By 1960, it was apparent that the thalassemias composed a heterogeneous group of genetic disorders. With the advent of molecular biology, many groups of researchers have widely studied thalassemias. Methods developed in the last 25 years have enabled researchers to measure the quantity of globin chains synthesized and identify specific genetic mutations.

Thalassemia is now recognized as one of the most common genetic disorders affecting the world's population. Approximately 1–5% of people are thought to be carriers of β-thalassemia.[3] It is estimated that between 100,000 and 200,000 individuals worldwide are born each year with severe forms of thalassemia, and approximately 60,000 of those have β-thalassemia.[3] In North America, about 20% of immigrants from Southeast Asia and 6–11% of African Americans have detectable α-thalassemia. Many more are silent carriers. About 6% of individuals with Mediterranean ancestry, 5% of Southeast Asians, and 0.8% of African Americans have β-thalassemia along with people from the Middle East and India.[4]

Thalassemia is a major health problem in countries where these disorders are prevalent. Prevention is seen as an essential part in the management of the problem. Thus, many of these countries now have large screening and education programs to detect carriers. This has drastically reduced the number of individuals born with both homozygous and heterozygous forms of the disease.

Thalassemia Versus Hemoglobinopathy

The system of categorizing thalassemias and hemoglobinopathies differs among hematologists. Some use hemoglobinopathy as a disease category to encompass both structural variants of hemoglobin (e.g., sickle cell anemia) and thalassemias. Others categorize only the structural variants as hemoglobinopathies and describe thalassemias as a separate disease entity. In this text, we refer to the two diseases as separate entities.

In the preceding chapter, *hemoglobinopathies* were defined as qualitative defects in the structure of globin chains resulting in production of abnormal hemoglobin molecules. Thalassemias, on the other hand, are typically quantitative disorders of hemoglobin synthesis that produce reduced amounts of normal hemoglobin.

The different clinical presentations of hemoglobinopathies and thalassemia are a direct result of the differences in the types of mutations encountered in these disease states. Most hemoglobinopathies result predominantly from a point mutation within a globin gene that is translated into a globin chain containing a single amino acid substitution. Hemoglobin types containing a point mutation in the globin may be produced at normal levels but are structurally abnormal. They can be unstable or have abnormal function. In contrast, thalassemias result from both deletional and nondeletional mutations in globin genes that reduce or eliminate the synthesis of the corresponding globin chain. This results in the assembly of inadequate amounts of normal hemoglobin and a reduced oxygen-carrying capacity of the blood. Unlike hemoglobinopathies, the amino acid sequence of the chain, if produced, is usually normal, and the chain is assembled into the appropriate hemoglobin, albeit in reduced amounts. In some of the less common thalassemias, the globin chains can be lengthened or truncated (Table 14-1 ★).

☑ CHECKPOINT 14-1

Differentiate the etiology of thalassemias and hemoglobinopathies.

★ **TABLE 14-1** Comparison of Hemoglobinopathies and Thalassemias

Disease	RBC Count	Indices	Erythrocyte Morphology	Abnormal Hb	Hb Solubility Test	Ancestry	Reticulocyte Count
Hemoglobinopathy	↓	Normocytic, normochromic	Target cells, sickle cells (in HbS), HbC crystals (in HbC), others	HbS, HbC, HbE, etc.	+ in HbS, Hb Bart's, and HbC^{Harlem}	African, Mediterranean, Middle Eastern, Southeast Asian	↑↑
Thalassemia	↑ Compared with what is expected for the Hb level	Microcytic, hypochromic	Target cells, basophilic stippling	HbH (β^4), Hb Bart's (γ^4)	Negative	Mediterranean, Southeast Asian, African	↑

↓ = slight decrease; ↑ = slight increase; ↑↑ = moderate increase; Hb = hemoglobin, RBC = red blood cell; + = positive

Genetic Defects in Thalassemia

Nearly all thalassemic mutations fall into one of five categories of genetic lesions: gene deletion, promoter mutation, nonsense mutation, mutated termination (stop) codon, and splice site mutation (Table 14-2 ★). Regardless of the type of mutation encountered, the results are the same: the globin chain encoded by the mutated globin gene is absent, reduced in concentration, or occasionally somewhat longer or shorter than normal.

If all globin genes of a single type of globin chain are affected, the corresponding hemoglobin is absent. Deleted genes are most common in α-thalassemia. Reduction in globin chain production from nondeletional mutations is more common in the β-thalassemias. The degree of reduction in globin chain production is a direct reflection of the type of mutation encountered and parallels the severity of the clinical disorder (Table 14-2).

☑ CHECKPOINT 14-2

What are the most common genetic mutations associated with α-thalassemia?

Types of Thalassemia

Because six different normal globin genes exist ($\alpha, \beta, \gamma, \delta, \epsilon, \zeta$), at least six versions of thalassemia are possible. In addition, deletions can occur to entire **gene clusters**, concurrently affecting more than one globin chain. Of the six normal globin genes, ϵ and ζ (ϵ, ζ) are normally synthesized only early in utero, and γ is produced in high amounts from approximately the third trimester of pregnancy until birth. Shortly before birth, γ-chain synthesis begins to decrease but can still be detected in low amounts in adult life in some people. The three remaining globin chains (α, β, and δ) along with the γ-chains are considered normal adult globin chains and combine to form hemoglobin A ($\alpha_2\beta_2$), hemoglobin A_2 ($\alpha_2\delta_2$), and hemoglobin F ($\alpha_2\gamma_2$), respectively. Approximately 97% of normal adult hemoglobin is HbA; thus, a deficiency of either α- or β-chains affects hemoglobin A assembly, reducing HbA concentration and affecting the blood's oxygen-carrying capacity.

Two major types of classical thalassemia, α-thalassemia and β-thalassemia, have been described. When synthesis of the α-chain is impaired, the disease is α-thalassemia. When synthesis of the β-chain is affected, the disease is β-thalassemia. There have been reports of δ-thalassemia, but its occurrence is rare and it is not clinically significant because the δ-chain is a component of the minor hemoglobin HbA_2, which comprises only ~2.5% of total hemoglobin. Combinations of gene deletions such as $\delta\beta$-thalassemia or $\gamma\delta\beta$-thalassemia occur but are rare. The synthesis of all the indicated chains is reduced.

Occasionally synthesis of a structural globin variant decreases globin chain synthesis, producing the clinical picture of thalassemia (thalassemic hemoglobinopathy). These structural variants include those hemoglobins with abnormally long or short globin chains (e.g., hemoglobin Constant Spring) as well as variants with a point mutation (e.g., hemoglobin E). Hemoglobin Lepore is a hemoglobin variant in which the non-α-globin chains are not only structurally abnormal but also ineffectively synthesized. Because of their clinical similarity to thalassemias, these particular structural variants are discussed in this section.

A variant of β-thalassemia known as hereditary persistence of fetal hemoglobin (HPFH) is characterized by continued production of increased amounts of HbF throughout life. This disorder is characterized by a failure in the switch of γ-chain production to β-chain production after birth. In homozygotes, 100% of circulating hemoglobin is HbF.

★ **TABLE 14-2** Five Common Genetic Defects in Thalassemia

Mutation Type	Thalassemia Encountered	Effect on Gene	Effect on Globin Chain
Deletion (large)	Predominantly α-thalassemia, some β-thalassemia	Loss of gene	Absence of production
Promoter	Predominantly β-thalassemia	Impaired transcription	Reduced or absent production
Nonsense	Predominantly β-thalassemia	In frame substitution	Amino acid change
		Frame shift	Amino acid changes distal to shift
			Longer or shorter globin chains
Stop codon	Predominantly β-thalassemia	Convert stop codon to amino acid codon	Slightly lengthened globin chain (retained)
			Significantly lengthened globin chain (degraded)
Splice site	Predominantly β-thalassemia	Create new splice sites	Slightly shortened globin chain (retained)
			Significantly shortened globin chain (degraded)
		Loss of splice sites	Slightly lengthened globin chain (retained)
			Significantly lengthened globin chain (degraded)
			Unaltered globin chain

Pathophysiology

Normally, equal amounts of α- and β-chains are synthesized by the maturing erythrocyte, resulting in a β-chain to α-chain ratio of 1:1. In α- and β-thalassemia, synthesis of one of these chains is decreased or absent, resulting in an excess of the other chain. If the α-chain is affected, there is an excess of β-chains, and if the β-chain is affected, there is an excess of α-chains. This unbalanced synthesis of chains contributes substantially to the pathophysiology in thalassemia and produces several effects, all of which contribute to anemia: (1) a decrease in total erythrocyte hemoglobin production, (2) ineffective erythropoiesis, and (3) chronic hemolysis.

The exact nature of the contribution of unbalanced chain production depends on which globin chain accumulates in excess and on the amount of that excess. There is no compensatory downward adjustment of production of one globin chain when its partner globin subunit is not synthesized normally. Although excess β- and γ-chains can combine as tetramers ($\beta 4$, $\gamma 4$) to form abnormal hemoglobins, α-chains do not. Excess α-chains are highly insoluble and precipitate within the cell. The precipitates (Fessas bodies) bind to the cell membrane, causing membrane damage (which leads to apoptosis) and decreased erythrocyte deformability. Macrophages may destroy the precipitate-filled developing erythroblasts in the bone marrow, resulting in a large degree of ineffective erythropoiesis. Cells that survive the marrow environment and enter the circulation also contain precipitates that are subsequently pitted and/or removed by the spleen, causing chronic extravascular hemolysis.

Excess β-chains can combine to form hemoglobin molecules containing four β-chains, hemoglobin H (HbH, β_4). This hemoglobin has a high oxygen affinity and is also unstable. Thus, it is a poor transporter of oxygen. In the fetus, when α-chains are decreased, excess γ-chains can combine to form hemoglobin molecules with four γ-chains, hemoglobin Bart's (Hb Bart's, γ_4). This hemoglobin also has a very high oxygen affinity.

Clinical Findings

Clinical findings are related to anemia, chronic hemolysis, and ineffective erythropoiesis (Table 14-3 ★). The combination of reduced HbA synthesis, ineffective erythropoiesis, and hemolysis results in anemia. The severity of anemia varies widely, depending on the specific genetic mutation and number of genes affected. Hypoxia from anemia is exacerbated in some cases by the presence of abnormal hemoglobins that have a high oxygen affinity (HbH and Hb Bart's). These hemoglobins do not release oxygen readily to the tissues.

Chronic hemolysis has several adverse effects. Splenomegaly is frequently present because the spleen is a major site of extravascular hemolysis. Occasionally, the spleen can become overburdened by the process of erythrocyte destruction resulting in **functional hyposplenism**. In this case, the spleen's function as a secondary lymphoid tissue is compromised, leading to an increase in infections (Chapter 7). Chronic hemolysis can also result in the formation of gallstones formed from the large amounts of bilirubin excreted by the liver. The chronic demand for erythrocytes also has adverse effects. The bone marrow responds by increasing erythropoiesis, resulting in erythroid hyperplasia, and in some of the more severe thalassemias, bone marrow expansion and thinning of calcified bone. Consequently, patients develop skeletal abnormalities and pathologic fractures. The increased iron demand needed

★ **TABLE 14-3** Clinical and Laboratory Findings Associated with Thalassemia

Clinical Finding	Pathophysiology	Laboratory Finding
Anemia/hypoxia	Decreased hemoglobin production/erythropoiesis	↓/N RBC count, ↓ hemoglobin, ↓ hematocrit
	Ineffective erythropoiesis	Microcytic/hypochromic RBCs
	Presence of high-affinity hemoglobins (HbH and Hb Bart's)	↓ MCV, ↓ MCH, ↓ MCHC
	Increased extravascular hemolysis	↑ Reticulocyte count
		Anisocytosis and poikilocytosis
		Target cells, basophilic stippling, nucleated RBCs
		BM erythroid hyperplasia
		↑ RDW
		Abnormal hemoglobin electrophoresis
Splenomegaly/hemolysis	Splenic removal of abnormal erythrocytes	↑ Bilirubin
	Ineffective erythropoiesis	↓ Haptoglobin
Gallstones	Increased intravascular and extravascular hemolysis	↑ Bilirubin
Skeletal abnormalities	Expansion of bone marrow	BM erythroid hyperplasia
Pathologic fractures	Thinning of calcified bone	
Iron toxicity	Iron overload	
	Multiple transfusions	↑ Prussian blue staining in BM
	Increased iron absorption	↑ Serum iron/ferritin and ↓ TIBC

BM = bone marrow; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; RBC = red blood cell; RDW = red cell distribution width; TIBC = total iron-binding capacity

to support the erythropoietic activity stimulates the increased absorption of iron, more than the amount required for erythropoiesis (Chapter 12). This additional iron is not effectively incorporated into hemoglobin, so it accumulates in macrophages in the bone marrow, liver, and spleen. As this process continues, iron eventually accumulates in parenchymal cells of various organs and adversely affects organ function. Iron toxicity commonly affects such organs as the liver, pituitary, heart, and bone, resulting in dysfunctions such as cirrhosis, hypogonadism and growth failure, arrhythmias and cardiomyopathies, and pathologic fractures, respectively.[5] The additional iron introduced through transfusion therapy exacerbates iron overload. Ineffective erythropoiesis in the bone marrow can be accompanied by **extramedullary erythropoiesis** in the liver and spleen. Extramedullary erythropoiesis can produce masses large enough to cause **compression syndromes** (Table 14-3).

Pregnant women with thalassemia have physiological demands that impact the developing fetus to a greater extent than the mother. A developing fetus can experience diminished growth, premature birth, and even intrauterine death if the mother's oxygen concentration falls below 70 mm Hg. Pregnant women who present with a hemoglobin level of between 9 and 10 g/dL (90–100 g/L) around the time of delivery are often given a blood transfusion to improve oxygen delivery. To avoid iron overload caused by the combination of transfusion therapy and increased iron absorption, pregnant women can be given deferoxamine (an iron chelator used to help eliminate excess iron) during and after the transfusion.[6]

☑ CHECKPOINT 14-3

Why do α- and β-thalassemia result in more clinically severe disease than other types of thalassemia?

Laboratory Findings

Peripheral blood findings provide clues to the disease (Table 14-3). Thalassemias are characterized by microcytic, hypochromic anemia with a decrease in MCV, MCH, and usually MCHC. The erythrocyte count is often normal or slightly decreased, but increased relative to the hemoglobin and hematocrit levels. The Mentzer Index can be helpful in differentiating thalassemia from iron-deficiency anemia (a common cause of microcytic hypochromic anemia) (Chapter 12).[7] The index is calculated by dividing the MCV (fL) by the RBC count ($\times 10^{12}$/L). If the result is <13, diagnosis favors thalassemia whereas if the result is >13, diagnosis favors iron deficiency. The RDW can be increased or within the reference interval. In iron deficiency, the RDW is most often increased (>15).[8] In practice, the CBC, Mentzer Index, and RDW should not be used as the only parameters to differentiate the two pathologies. In microcytic hypochromic anemia, iron studies (e.g., serum ferritin) should be performed routinely. Target cells and microcytosis usually are present even in cases without anemia. Basophilic stippling and nucleated erythrocytes can be present. Anisocytosis and poikilocytosis are common. Precipitates of excess chains or unstable hemoglobin can be visualized with supravital stains. Reticulocytes and bilirubin are usually increased due to the chronic hemolysis, whereas haptoglobin can be decreased, depending on the degree of intravascular hemolysis.

Hemoglobin electrophoresis is always indicated if thalassemia is suspected. HbA is usually decreased. HbF and HbA_2 are increased in β-thalassemia but decreased in α-thalassemia. Hemoglobin Bart's and HbH can also be present in some of the α-thalassemia syndromes.

Bone marrow studies are not necessary for diagnosis but when performed show marked erythroid hyperplasia. Erythroblasts appear abnormal with very little cytoplasm, uneven cytoplasmic membranes, and striking basophilic stippling. Prussian blue stain reveals an abundance of iron and occasionally a few ringed sideroblasts. Phagocytic "foam" cells similar to Gaucher cells have been reported in the more severe forms of the disease. This morphology results from partially digested red cell membrane lipids associated with intense ineffective erythropoiesis.

In areas of high prevalence of thalassemia and in certain populations, screening programs to detect thalassemia carriers have been developed. Thus, assays that are uncomplicated, time efficient, and accurate need to be used. A variety of PCR-based nucleic acid assays can be performed to identify mutations in thalassemia, including allele-specific oligonucleotide hybridization (ASO), dot blot and reverse dot blot assays, amplification refractory mutation system (ARMS), and direct sequencing[9] (Chapter 43). Given the large number of mutations present in the human globin genes, a small panel of probes for mutations found in a specific ethnic group are initially screened for together with the wild-type allele. Using this approach, identification of the mutation(s) is achieved $>90\%$ of the time. In cases where the mutation(s) are not identified, a second round of multiplex screening can be performed using a panel of more rare mutations; this is successful in most of the remaining 10% of cases. Gene sequencing can be performed to identify mutations that evade both rounds of screening.[10] The dot blot methodology is used to identify a specific mutation when one has been identified in a specific kindred as opposed to screening for unknown mutation(s).[11]

With the development of capillary sequencing techniques, PCR amplified target genes can be sequenced and compared with wild-type globin DNA to detect mutations. Until recently, direct sequencing was expensive and labor intensive, so hybridization methods for nucleic acid analysis were favored. Direct sequencing is now a common method of mutation identification.

Nucleic acid-based methods are being developed for prenatal diagnosis of thalassemias and hemoglobinopathies. Extracellular fetal DNA is present in the maternal circulation. If the father is a known heterozygote for a particular thalassemia mutation that the mother does not have, detection of this allele in the maternal circulation confirms inheritance of the paternal gene by the child.[12]

α-THALASSEMIA

General Considerations

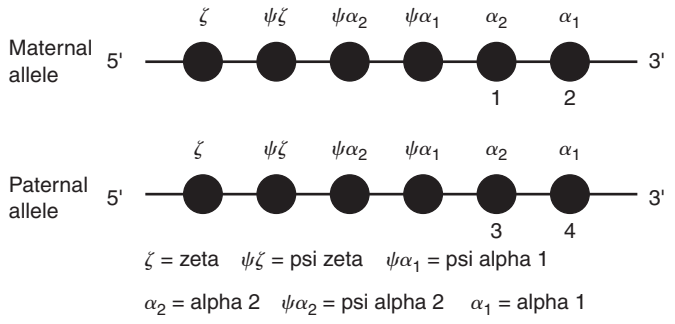
α-Thalassemia is a group of four disorders characterized by decreased synthesis of α-chains. Although each is discussed separately, several features common to each type are presented here.

Etiology

In the human genome, two α-genes are located on each of the two #16 chromosomes, totaling four α-genes in the **diploid** state (Figure 14-1 ■). Mutations can affect one or more of the α-genes, resulting in four discrete clinical severities. A patient in whom all four α-genes are deleted produces no α-chains, a condition referred to as *hydrops fetalis*. When three of the four α-genes are deleted, the disorder is known as *hemoglobin H* (*HbH*) *disease*. The deletion of two α-genes is known as α-thalassemia *minor*, and the deletion of a single α-gene is known as *silent carrier*. Though less common, nondeletional mutations and mutations that produce unstable α-chains are also found in α-thalassemia. The outcome is usually the same as that of a deletion mutation, a reduction in α-chains and in the corresponding α-containing hemoglobins.

Affected Alleles

The amount of α-chains synthesized is somewhat proportional to the number of affected **alleles**. However, sometimes erythrocytes produce higher concentrations of α-chains than the number of affected alleles would predict. There are two main reasons for this phenomenon. First, the two α-genes on each of the #16 chromosomes are designated as α_1 and α_2, with the α_2-gene positioned upstream (5′) of the α_1-gene (Figure 14-1). The α_2-gene produces two to three times the amount of mRNA as the α_1-gene.[13] Therefore, a deletion of the α_2-gene would reduce α-chain production to a greater degree than would a deletion of the α_1-gene. Second, the erythropoietic system has an internal mechanism designed to stimulate increased production of α-chains from the unaffected genes to compensate for deletions, thus lessening the net reduction of α-chains.



■ **FIGURE 14-1** A short section of chromosome 16 showing the 5′ to 3′ orientation of three functional genes ζ, α_2, and α_1 along with three pseudogenes ψζ, ψα_2, and ψα_1. Pseudogenes are the result of partial gene duplications but are not expressed. There are two functional α-genes on each chromosome; the α_2-gene expresses 2–3 times as much protein product as the α_1-gene.

Affected Individuals

α-thalassemia is found primarily in people of Mediterranean, Asian, and African ancestry. In particular, it is commonly seen in blacks, Indians, Chinese, and Middle Eastern people, with blacks usually expressing a milder version of the disease. The reason patients of African descent tend to present with a milder version of α-thalassemia is because the deletion in this ethnic group usually involves the lower-producing α_1-gene.

Genotypes

Three nomenclature systems—genotypic, genotypic description, and phenotypic—have been developed to classify the α-thalassemias into five discrete categories. The addition of the normal **genotype** produces a total of six possibilities. The genotypic system designates deleted genes as (−) and unaffected genes as (α). The genotypic description system combines the **zygosity** state, **homozygous** or **heterozygous**, with either a gene symbol (α^0 or α^+) or a nominal descriptor (α-thal-1 or α-thal-2) to designate the number of deleted α-genes on each chromosome. Both α-thal-1 and α^0 indicate the deletion of both α-genes on the same chromosome (cis deletion) (−, −). α-thal-2 and α^+ refer to one deleted and one unaffected α-gene on a given chromosome (−, α). The phenotypic system describes four clinical types, hydrops fetalis, hemoglobin H disease, α-thalassemia minor, and silent carrier with the α-thalassemia minor type exhibiting two clinical severities (Table 14-4 ★).[13] About 15–20% of patients have a nondeletional mutation of the α-thalassemia gene, designated as α^T, that functions to reduce but not eliminate α-chain production from that gene.

α-Thalassemia Major (α^0/α^0 or α-thal-1/α-thal-1; Hydrops Fetalis)

The most severe form of α-thalassemia, α-thalassemia major, involves the deletion of all four α-genes (− −/− −). Both parents of the thalassemia patient must have α-thalassemia to have a child with hydrops fetalis because both α-genes on each parental chromosome inherited by the child are deleted (α-thal-1; −, −). α-Thalassemia major is found almost exclusively in Asians because that is the major ethnic group carrying the α-thal-1 allele.

Pathophysiology

Because all four α-genes are deleted in hydrops fetalis, no physiologically useful hemoglobins can be synthesized beyond the embryonic state. Therefore, this disorder is incompatible with life, and infants are either stillborn or die within hours of birth. In the absence of α-chains, erythrocytes assemble hemoglobin using the γ-, δ-, and β-chains available. Therefore, abnormal hemoglobin tetramers involving γ-chains (Hb Bart's, γ_4) are produced. Hb Bart's has a very high oxygen affinity and no Bohr effect (Chapter 6). Therefore, this hemoglobin cannot supply tissues with sufficient oxygen to sustain life, and the developing infant usually dies of hypoxia and congestive heart failure in utero. Hemoglobin Portland, although normally absent following the first trimester, continues to be synthesized until birth in α^0/α^0 thalassemia because it does not contain α-chains.

★ **TABLE 14-4** Characteristics of α-Thalassemia

Genotype	Genotypic Description	Phenotype	Hematologic Findings	Severity	Hemoglobins Present
(− −/− −) α^0/α^0	Homozygous α-thal-1	Hydrops fetalis	Marked anemia Microcytic/hypochromic RBCs ↑↑↑ anisopoikilocytosis ↑ NRBC	Fatal	Hb Bart's (80–90%) Hb Portland (10–20%)
(− −/−α) α^0/α^+	Heterozygous α-thalassemia-1/ α-thalassemia-2	Hemoglobin H disease	Moderate to marked anemia Microcytic/hypochromic RBCs Target cells Basophilic stippling Poikilocytosis	Chronic, moderately severe hemolytic anemia	Birth = Hb Bart's; Adult = HbH
(− −/αα) α^0/α	Heterozygous α-thalassemia-1	α-thalassemia-minor	Slight anemia Microcytic/hypochromic RBCs Target cells Basophilic stippling Poikilocytosis	Mild to moderate	Birth = Hb Bart's; Adult = normal
(−α/−α) α^+/α^+	Homozygous α-thalassemia-2	α-thalassemia-minor	Slight anemia Microcytic/hypochromic RBCs Target cells Basophilic stippling Poikilocytosis	Mild	Birth = Hb Bart's; Adult = normal
(−α/αα) α^+/α	Heterozygous α-thalassemia-2	Silent carrier	Normocytic or slightly microcytic RBCs	Normal	Normal
(αα/αα)	Normal	None	Normal	Normal	Normal

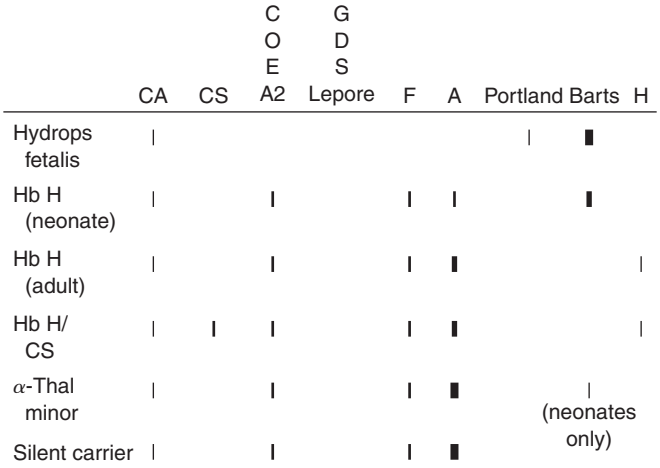
↑↑↑ = marked increase; ↑ = slight increase

Clinical Findings

Infants who survive until birth exhibit significant physical abnormalities upon routine exam. The babies are underweight and edematous with a distended abdomen. The liver and often the spleen are enlarged due to extramedullary hematopoiesis. There is massive bone marrow hyperplasia. Hemolysis in the fetus is severe and there is extensive deposition of hemosiderin.

Laboratory Results

Laboratory results confirm the clinical observation of severe anemia with hemoglobin values ranging from 3–10 g/dL (30–100 g/L) and erythrocytes that are markedly microcytic and hypochromic. Hemoglobin electrophoresis on cellulose acetate or agarose at alkaline pH shows 80–90% Hb Bart's and 10–20% Hb Portland; HbH is sometimes also detectable. HbA, HbA_2, and HbF are absent due to the lack of α-chain production (Figure 14-2 ■).



■ **FIGURE 14-2** Hemoglobin electrophoresis on cellulose acetate or agarose at pH 8.4 is helpful in distinguishing the type of thalassemia and in differentiating thalassemias from hemoglobinopathies. In α-thalassemias, there is a reduction in α-containing hemoglobins (HbA, HbA_2, and HbF) proportional to the number of deleted α-genes and in the more severe cases, the emergence of non-α-containing hemoglobins (HbH and Hb Bart's).

☑ CHECKPOINT 14-4

Which of the three normal adult hemoglobins would be affected in hydrops fetalis?

Hemoglobin H Disease (α^0/α^+ or α-thal-1/α-thal-2)

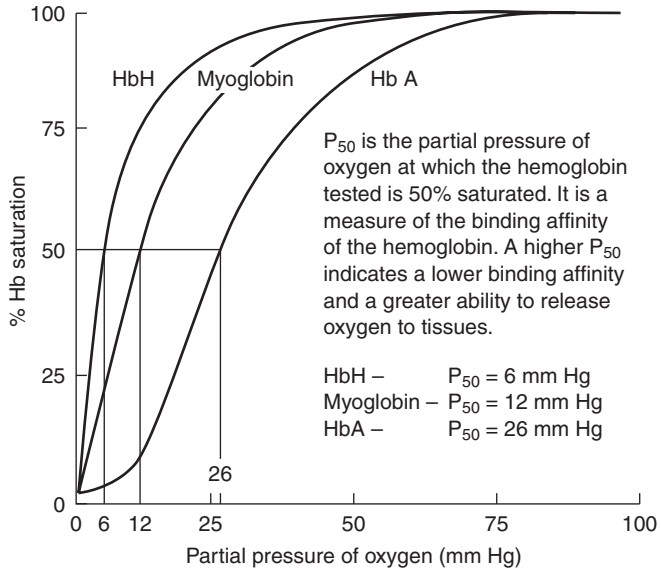
HbH disease, a symptomatic but nonfatal type of α-thalassemia, was the first type to be described in 1956. It occurs when three of the four α-genes are deleted ($-,-/-,\alpha$). African Americans seldom present with HbH disease because they rarely express a deletion of two α-genes on the same chromosome.[14]

This disorder usually results when two parents, one with heterozygous α-thal-1 ($--/\alpha\alpha$) and the other with the heterozygous α-thal-2 ($-\alpha/\alpha\alpha$) genotype, bear children.[13] All children from a patient with HbH disease will have a type of α-thalassemia, the severity of which depends on the allele inherited and the other parent's genotype.

Pathophysiology

The dramatic reduction in α-chain synthesis (25–30% of normal) results in a decrease in the assembly of HbA, HbA_2, and HbF. In addition, a decrease in α-chains creates a relative excess of β-chains, which assemble to form β-chain tetramers called HbH. γ-Chains also are produced in excess of α-chains, especially at birth, and combine to form γ-chain tetramers or Hb Bart's.

HbH is thermolabile, unstable, and tends to precipitate inside erythrocytes triggering chronic hemolytic anemia. Its oxygen affinity is 10 times that of HbA, reducing oxygen delivery to the tissues. Its high oxygen affinity is attributed to the lack of heme–heme interaction and absence of the Bohr effect (Chapter 6). This increased oxygen affinity is reflected in the lower **P_{50} value** of HbH relative to HbA and myoglobin (Figure 14-3 ■).



■ **FIGURE 14-3** The Hb dissociation curve illustrates the relative binding affinities of HbA, HbH, and myoglobin using the P_{50} value. The monomeric myoglobin molecule lacks heme–heme interactions, causing it to bind oxygen tightly, decreasing the P_{50} value relative to HbA. The P_{50} value is even lower for HbH, indicating an even stronger affinity for oxygen estimated to be 10 times more than the oxygen affinity of HbA.

Hemoglobin H can also occur as an acquired defect in erythroleukemia and other myeloproliferative neoplasms. However, the clinical manifestations and hematological abnormalities of these acquired disorders make it possible to distinguish them from HbH disease. Acquired HbH is probably due to a defect that prohibits the transcription of the α-gene.

☑ CHECKPOINT 14-5

Compare oxygen-binding characteristics of HbH relative to HbA and myoglobin.

Clinical Findings

Symptoms are related to anemia and chronic hemolysis. Hemoglobin H disease shows a wide variation from mild to severe anemia, which worsens during pregnancy, in infectious states, and during administration of oxidant drugs. Splenomegaly and, less often, hepatomegaly are present. Less than half of affected patients exhibit skeletal changes similar to those found in β-thalassemia major, and there is relatively little ineffective erythropoiesis.

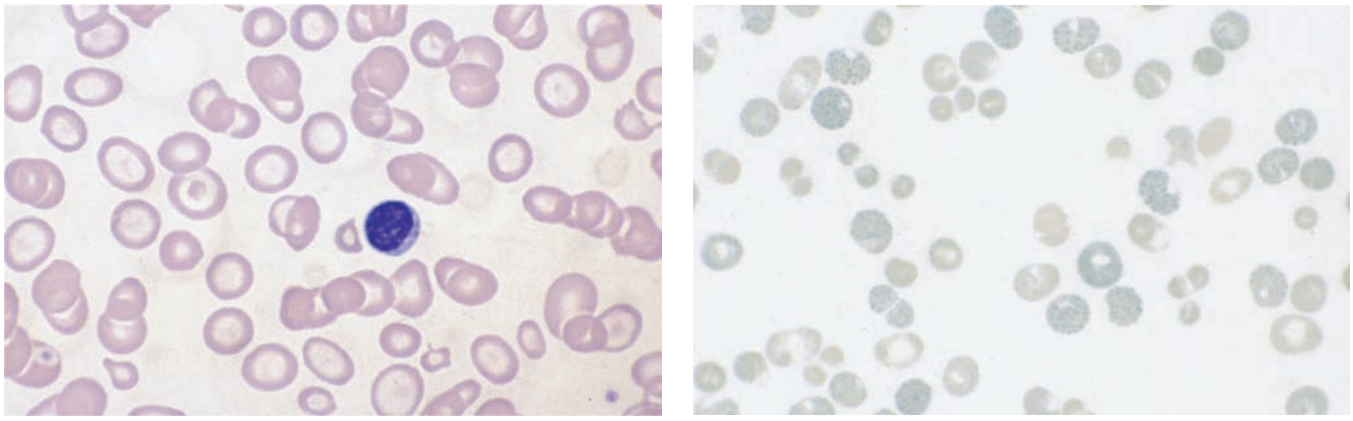
Laboratory Results

Hemoglobin H disease is characterized by a microcytic, hypochromic anemia with hemoglobin levels usually ranging from 8–10 g/dL. Reticulocytes are moderately increased to 5–10%, and nucleated red blood cells are observed on the peripheral blood smear (Figure 14-4a ■).

Hemoglobin electrophoresis of affected neonates shows about 25% Hb Bart's with decreased levels of HbA, HbA_2, and HbF. Shortly before birth, β-chains begin to replace γ-chains, and HbH eventually replaces Hb Bart's. Hemoglobin H, a fast-migrating hemoglobin at alkaline pH, constitutes 2–40% of the hemoglobin in adults with HbH disease. HbA_2 is decreased to about 1.5%, but HbF is normal. A trace of Hb Bart's can be demonstrated in approximately 10% of affected adults with remaining hemoglobin being HbA (Figure 14-2). Other laboratory tests are available to assess patients with HbH disease. Hemoglobin H inclusions are easily found upon incubation of blood with brilliant cresyl blue (Figure 14-4b). These inclusions tend to cover the inside of the plasma membrane, giving the appearance of a golf ball.

Treatment and Prognosis

Treatment for patients with HbH disease is variable but in severe cases can involve long-term transfusion therapy and splenectomy. Regular transfusions minimize the stunting of growth and the other consequences of chronic severe anemia. Iron chelation therapy with deferoxamine avoids iron overload and the effects of iron toxicity. Deferaserox (licensed in the United States in 2006) and deferipone (not yet licensed in the United States) are oral chelators that serve as an alternative to deferoxamine but have significant dose-related sequelae including neutropenia, arthropathy, and agranulocytosis.[4] The increased and ineffective erythropoiesis places high demands on the bone marrow requiring folate supplementation to avoid a deficiency.[5] Early treatment is necessary to prevent the typical clinical



a b

■ **FIGURE 14-4** **(a)** This peripheral blood smear is from a patient with HbH disease. Note the microcytic, hypochromic anemia with target cells (Wright-Giemsa stain; 1000× magnification). **(b)** Peripheral blood from patient in Figure 14-4a after incubation with brilliant cresyl blue. Notice the cells that have dimples and look like golf balls. These are the cells with precipitated HbH. (Brilliant cresyl blue stain; 1000× magnification).

manifestations of thalassemia. With supportive care and behavioral interventions, patients with HbH disease experience a normal life expectancy. For patients in whom transfusion and chelation therapy are not efficacious, a bone marrow transplant could be indicated.[5]

α-Thalassemia Minor (α-thal-2/α-thal-2 [a^+/a^+], or α-thal-1/normal [α^0/α])

The α-thalassemia trait (homozygous α-thal-2 or α-thal-1 trait) occurs when two of the four α-genes, either on the same (cis) or opposite (trans) chromosomes, are missing. The condition is found in all geographic locations.[15] In African Americans, the homozygous α-thal-2 form is the most common presentation. In individuals of Southeast Asian or Mediterranean descent, genetic testing has identified at least nine **haplotypes** (groups of alleles of different genes on the same chromosome that are closely linked and usually inherited together), representing different mutations, all of which result in the deletion of both α-genes on the same chromosome.[16]

Pathophysiology

Although a measurable decrease in the production of α-containing hemoglobins occurs, the unaffected α-globin genes are able to direct synthesis of α-globin chains to a greater than normal degree and therefore partially compensate for the deleted genes. Only minor changes occur in the erythrocyte count, indices, hemoglobin electrophoresis patterns, and red cell morphology.

Clinical Findings

Patients with α-thalassemia trait are asymptomatic with a mild anemia and are often diagnosed incidentally or when being evaluated for family studies. This mild **phenotype** is the reason that this form is called *thalassemia minor*.

Laboratory Results

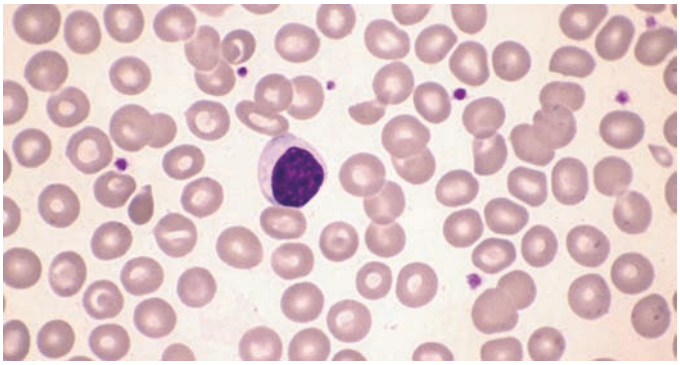
The most demonstrable laboratory abnormalities are observed in the newborn. The presence of 5–6% Hb Bart's in neonates can be helpful in diagnosing this condition.[17] Three months after birth, Hb Bart's decreases to undetectable levels and hemoglobin electrophoresis becomes normal. The only persistent hematological abnormality thereafter is a mild microcytic, hypochromic anemia.

In adult patients, hemoglobin levels are above 10 g/dL (100 g/L), and the erythrocyte count is above 5×10^{12}/L. The peripheral blood film usually demonstrates significant microcytosis with an MCV of 60–70 fL with few target cells (Figure 14-5 ■). Occasional cells can exhibit HbH inclusions after incubation with brilliant cresyl blue.

In some cases, α-thalassemia may be masked by iron-deficiency anemia. Persistence of microcytes following successful treatment of iron deficiency is suggestive of thalassemia, but further investigation is needed for conclusive diagnosis.

Treatment and Prognosis

These patients are usually asymptomatic, have a normal life span, and do not require medical intervention for their thalassemia.



■ **FIGURE 14-5** This is a peripheral blood smear from a patient with α-thalassemia trait. The hemoglobin is 15 g/dL, RBC count 6.4×10^{12}/L, MCV 69.4 fL. The high RBC count with microcytosis is typical of thalassemia minor or trait (peripheral blood; Wright-Giemsa stain; 1000× magnification).

Silent Carrier (α-thal-2/normal; α^+/α)

The silent carrier version of α-thalassemia (α-thal-2 trait) is missing only one of four functioning α-genes. More than 25% of African Americans have been shown to express a deletion of one α-gene.[18–20]

Pathogenesis/Clinical Findings/Laboratory Results

In the silent carrier state, the three remaining α-genes direct the synthesis of an adequate number of α-chains for normal hemoglobin synthesis. This carrier state is asymptomatic and benign, but adults often present with a borderline normal MCV of around 78–80 fL.[16] In affected infants, 1–2% Hb Bart's may be found at birth but cannot be detected after three months of age. The only definitive diagnostic test for thalassemias in adults with one or two gene deletions is globin gene analysis.

Treatment and Prognosis

Patients with the silent carrier phenotype require no treatment and have a normal life span.

CASE STUDY (continued from page 252)

The parents took John, the 4-year-old Greek patient, to a pediatrician for a checkup. A CBC ordered had the following results:

CBC		Differential	
WBC	11.4 × 10^9/L	Segs	55%
RBC	1.7 × 10^{12}/L	Bands	1%
Hb	8.3 g/dL	Lymphs	36%
Hct	0.24L/L	Mono	7%
MCV	69 fL	Eos	1%
MCH	21 pg	Moderate poikilocytosis, poly-	
MCHC	29.2 g/dL	chromasia, and many target	
Plt	172 × 10^9/L	cells; few teardrop cells	

1. Based on the indices, classify the anemia morphologically.
2. Name the dominant poikilocyte observed in this peripheral blood smear.
3. Name three disorders that frequently present with the same poikilocyte that dominates in this peripheral blood smear.
4. List two additional lab tests that would help to confirm the diagnosis and predict the results of each.

β-THALASSEMIA

General Considerations

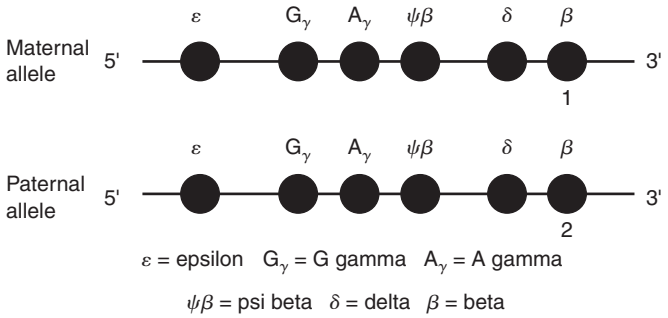
As with α-thalassemias, some features of β-thalassemias are common to all forms of the disease. The genetics of β-thalassemia and the individuals affected with the disorder are presented before the discussion of its various forms.

Genetics

Whereas a total of four α-globin genes results in four major genotypes of α-thalassemia, there are only two β-globin genes, one located on each chromosome 11 (Figure 14-6 ■). If the prominent type of mutation found in β-thalassemia were also deletional, one would expect two severities, the severe homozygote and the mild heterozygote. However, in β-thalassemia, most mutations are nondeletional, resulting in a near continuum of clinical severities. Two classification systems are currently used for this diverse group of diseases. The genotypic system classifies β-thalassemia patients into six genotypes based on zygosity and the degree of alteration of the β-genes, and the phenotypic system divides patients into four categories based on the severity of clinical symptoms.

In the genotypic system, all β-gene mutations are categorized into two groups based on the impact of the mutation on β-globin production. The two gene varieties are termed β^+ and β^0. The β^+-gene mutation causes a partial block in β-chain synthesis, and the β^0-gene mutation results in a complete absence of β-chain synthesis from that allele. In addition, a minimally affected β-allele called *silent carrier* (β^{SC}) has been identified. It is found only in the most benign version of β-thalassemia. In the heterozygous state, hematological features are normal; it is clinically benign. Diagnosis can be made only by finding a slight imbalance of α-/non-α-chain synthesis.[9] When the three gene designations, β^0, β^+, and β^{SC} are combined with the normal allele (β), and the two possible zygosity patterns (homozygous and heterozygous) are considered, eight possible genotypes (β^o/β^o, β^o/β^+, β^+/β^+,/β^o/β, β/β^+, β/β, β/β^{SC}, β^{SC}/β^{SC}) emerge (Table 14-5 ★).

β-thalassemia is the result of several different types of molecular defects. More than 200 mutations resulting in partial to complete absence of β-gene expression have been described, but only 20 mutations account for 80% of the diagnosed β-thalassemias.[1,14] β-thalassemia is rarely due to deletion of the structural gene as is the case in the α-thalassemias. Most defects in β-thalassemia are point mutations in regions of the DNA that control β-gene expression. These types of mutations can affect gene expression ranging from minor reductions in β-globin production to complete absence of synthesis. Mutations can affect any step in the pathway of globin gene expression, including gene transcription, RNA processing, mRNA translation, and post-translational integrity of the protein.[21,22]



■ **FIGURE 14-6** Chromosome 11 is the location of four types of globin genes (ϵ, γ, δ, β). The 5′ to 3′ orientation of the genes is depicted. There is a gene for ϵ, δ, and β and two γ-genes. A β-pseudogene ($\psi\beta$) has been identified but does not express protein product.

★ **TABLE 14-5** Characteristics of β-Thalassemia

Genotype	Zygosity	Phenotype	RBC Count	RBC Morphology	Hb Electrophoresis	Severity
β^0/β^0	Homozygous	Major	Relative ↑	↑↑↑ Target cells	No A, ↑ A_2, ↑↑ F	Severe
β^0/β^+	Double heterozygous	Major	Relative ↑	↑↑↑ Target cells	↓↓ A, ↑ A_2, ↑↑ F	Severe
		Intermedia		↑↑ Target cells	↓ A, ↑ A_2, ↑↑ F	Moderate
β^+/β^+	Homozygous	Major	Relative ↑	↑↑↑ Target cells	↓↓ A, ↑ A_2, ↑↑ F	Severe
		Intermedia		↑↑ Target cells	↓ A, ↑ A_2, ↑↑ F	Moderate
β^0/β	Heterozygous	Intermedia	Relative ↑	↑↑ Target cells	↓ A, ↑ A_2, ↑ F	Moderate
		Minor		↑ Target cells	↓ A, ↑ A_2, ↑ F	Mild
β^+/β	Heterozygous	Minor	Relative ↑	↑ Target cells	↓ A, ↑ A_2, ↑ F	Mild
β^{SC}/β^{SC}	Homozygous	Mild Intermedia	Relative ↑	↑Target cells	↓ A, ↑ A_2, ↑ F	Mild
β^{SC}/β	Heterozygous	Minima	Normal	± Target cells	Normal	Normal
β/β	Homozygous	Normal	Normal	Normal	Normal	Normal

↑ = slight increase; ↑↑ = moderate increase; ↑↑↑ = marked increase; +/− = occasionally seen; ↓ = slight decrease; ↓↓ = moderate decrease; A = HbA, A_2 = HbA_2; F = HbF; β^{SC} = silent carrier

Within a given population, a few genetic lesions account for most of the β-thalassemia mutations. For instance, in Greece, five mutations account for 87% of the gene defects.[22]

The phenotypic classification recognizes four groups of patients categorized by the severity of symptoms, medical interventions, and prognoses. The four groups, listed in order from most severe to least severe, are β-thalassemia major, β-thalassemia intermedia, β-thalassemia minor, and β-thalassemia minima (Table 14-5).

The phenotypic classification does not accurately reflect the genetic description of the disease. However, a disadvantage to the genotypic system is that patients with identical genotype designations can express β-thalassemia that is phenotypically diverse. For instance, a severe form of β^+/β^+-thalassemia (Mediterranean form) is characterized by an increase in HbF (50–90%) and a normal or only slightly elevated HbA_2, whereas a milder form of β^+/β^+-thalassemia (Black form) has 20–40% HbF with normal or elevated HbA_2, and the remainder HbA. For this reason, some clinicians prefer the phenotypic system that more closely parallels symptoms and better predicts clinical interventions necessary for appropriate management of the patient.

Affected Individuals

The most severe mutation (β^0) is found more frequently in the Mediterranean regions—specifically in northern Italy, Greece, Algeria, Saudi Arabia—and Southeast Asia. Two severities of the β^+ mutation tend to originate in different ethnic populations. The more severe version is observed in the Mediterranean region, the Middle East, the Indian subcontinent, and Southeast Asia; the milder version is localized to patients of African descent.[15]

β-Thalassemia Major (β^0/β^0, β^0/β^+, β^+/β^+)

Expected Genotypes

β-Thalassemia major, also referred to as *Cooley's anemia*, is caused by a homozygous (β^0/β^0, $\beta^+\beta^+$) or **double heterozygous** ($\beta^0\beta^+$) inheritance of abnormal β-genes resulting in marked reduction or absence of β-chain synthesis. As can be seen in Table 14-5, two of these three genotypes ($\beta^0\beta^+$, $\beta^+\beta^+$) can also present as the milder β-thalassemia intermedia phenotype that will be discussed subsequently.

Pathophysiology

The dramatic reduction or absence of β-chain synthesis affects the production of HbA. The symptoms that result from β-thalassemia major begin to manifest in infants approximately six months after birth. Other non-β-containing hemoglobins, HbA_2 and HbF, are increased in partial compensation for the decreased HbA levels.

The pathophysiologic mechanisms that result from a lack of β-chain production can be classified into four categories (Figure 14-7 ■):

- reduced HbA
- compensatory production of other hemoglobins
- ineffective erythropoiesis with hemolysis
- erythroid hyperplasia

A dramatic reduction in HbA compromises the blood's oxygen-carrying capacity. Other non-β-containing hemoglobins, HbF and HbA_2, are increased. HbF has a higher affinity for oxygen than HbA (Chapter 6). The result is to exacerbate the already compromised oxygen delivery to tissues.

In β-thalassemia major reduced synthesis of β-chains results in an excess of free α-chains and a β-to-α chain ratio of <0.25. The excess free α-chains cannot form hemoglobin tetramers, so they precipitate within the cell, damaging the cell membrane, and leading to chronic hemolysis.[23] The accumulating α-chains contain free iron and hemichromes that generate reactive oxygen species (ROS). The ROS damage hemoglobin as well as the membrane proteins and lipids, decreasing membrane stability.[24,25] Oxidation of membrane Band 3 produces clustering of the proteins, creating new antigens on the cell surface that bind IgG and complement.[26]

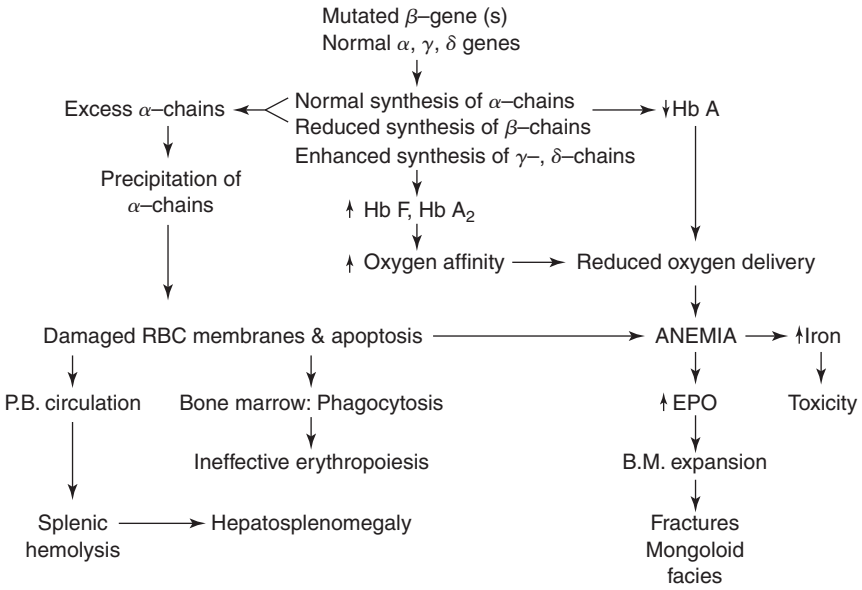


FIGURE 14-7 In β-thalassemia major, the decreased synthesis of β-chains reduces the production of HbA and increases the production of non-β-chain containing hemoglobins (HbA_2 and HbF). Excess α-chains form insoluble precipitates inside erythrocytes, damaging the membranes and reducing RBC lifespan through splenic sequestration and ineffective erythropoiesis. All of these factors contribute to a reduced oxygen delivery to the tissues resulting in anemia and hypoxia. The compensatory erythroid hyperplasia in the bone marrow expands the marrow cavity, resulting in pathologic fractures and mongoloid facial features.

Many IgG- and complement-sensitized erythrocytes in the bone marrow are destroyed by binding to marrow macrophages via Fc receptors, resulting in phagocytosis and a large degree of hemolysis.

The majority of the hemolysis occurs within the bone marrow primarily at the polychromatophilic erythroblast stage of erythroid development, resulting in ineffective erythropoiesis.[27] The ineffective erythropoiesis is due to activation of apoptotic mechanisms by the precipitated α-globin chains and damaged cellular components.[28,29] If the patient has inherited α-thalassemia with β-thalassemia, the symptoms associated with hemolysis may be reduced because the relative excess of α-chains is reduced by the co-inherited α-thalassemia.

The combination of reduced HbA, increased HbF, ineffective erythropoiesis, and chronic hemolysis results in significant anemia. The body attempts to compensate by stimulating erythropoiesis. The resulting erythroid hyperplasia causes bone marrow expansion and thinning of calcified bone. Increased erythropoietic activity also stimulates the absorption of more iron in the gut, leading to iron toxicity. Ineffective erythropoiesis in the bone marrow can be accompanied by extramedullary hematopoiesis in the liver and spleen, often producing hepatosplenomegaly (Figure 14-7).

☑ CHECKPOINT 14-6

Why are the symptoms of β-thalassemia major delayed until approximately the sixth month of life?

Clinical Findings

Early symptoms of β-thalassemia first observed in infants include irritability, pallor, and a failure to thrive and gain weight, beginning at about 6 months of age. Diarrhea, fever, and an enlarged abdomen are also common findings. If therapy does not begin during early childhood, the clinical picture of thalassemia develops within a few years.

Severe anemia is the clinical condition responsible for many problems experienced by these children. The anemia places a tremendous burden on the cardiovascular system as it attempts to maintain tissue perfusion. Constant high output of blood usually results in cardiac failure in the first decade of life and is the major cause of death in untreated children. Growth is retarded, and a bronze pigmentation of the skin is notable. Chronic hemolysis often produces gallstones, gout, and icterus.

Bone changes accompany the hyperplastic marrow. Marrow cavities enlarge in every bone, expanding the bone and producing characteristic bossing of the skull, facial deformities, and "hair-on-end" appearance of the skull on x-ray (Figure 14-8 ■). The thinning cortical bone in long bones can lead to pathologic fractures.

Extramedullary hematopoiesis can be found in the liver and spleen and occasionally elsewhere in the body. The spleen can become massively enlarged and congested with abnormal erythrocytes.

Other clinical findings are associated with the body's attempt to increase erythrocyte production. Features that suggest an increased metabolic rate include fever, lethargy, weakened musculature, decreased body fat, and decreased appetite. Infection is a common cause of death. Folic acid deficiency can develop as a consequence of increased utilization by the hyperplastic marrow.

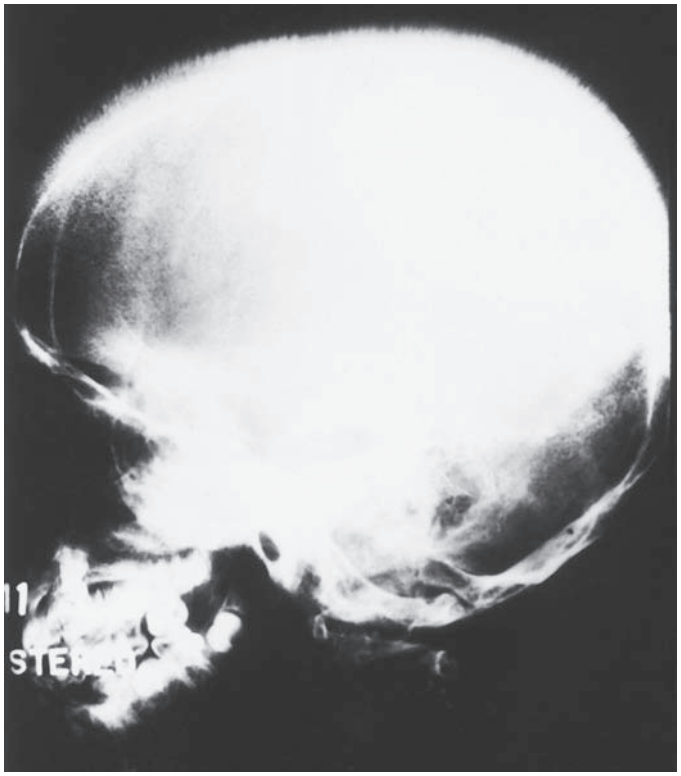


FIGURE 14-8 Increased erythropoiesis in the bone marrow of patients with β-thalassemia major expands the marrow cavity producing the typical "hair-on-end" appearance as seen on this radiograph of the skull of a boy with β-thalassemia.

Laboratory Results

The hemoglobin level can be as low as 2 or 3 g/dL (20–30 g/L) in the more severe forms of the disease. The anemia is markedly microcytic and hypochromic with an MCV of <67 fL[30] and a markedly reduced MCH and MCHC. The peripheral blood smear shows marked anisocytosis and poikilocytosis (Figure 14-9 ■). Precipitates of α-chains can be visualized with methyl violet stain. Variable basophilic stippling and polychromasia are noted. Reticulocytes are not increased to the degree expected for the severity of the anemia because of the high degree of ineffective erythropoiesis. Nucleated erythrocytes are almost always found, and the RDW can be normal or increased.[31] Secondary leukopenia and thrombocytopenia can be produced because these components also become trapped in the enlarged spleen. Chronic hemolysis is reflected by increased unconjugated bilirubin. Urine can appear dark brown from the presence of dipyrroles.

Bone marrow studies are not usually necessary for diagnosis but when performed show marked erythroid hyperplasia with an M:E ratio of 1:10 or less.

Hemoglobin electrophoresis performed on cord blood samples provides evidence of deficient β-chain production at birth. Although normal cord blood contains about 20% HbA, cord blood from infants with β-thalassemia major has <2% HbA.[21] In adults, hemoglobin electrophoresis shows variable results, depending on the thalassemia alleles inherited. Absence of HbA, 90% HbF, and low, normal, or increased HbA_2 is characteristic of β^0/β^0-thalassemia.[32]

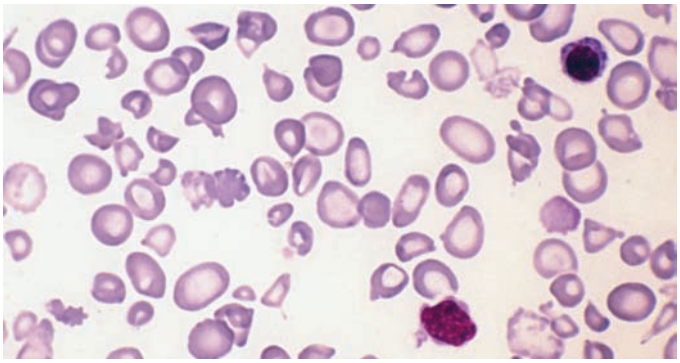


FIGURE 14-9 Peripheral blood smear from a patient with β-thalassemia major showing marked anisopoikilocytosis. Target cells, schistocytes, teardrops, and ovalocytes are the major poikilocytes observed. An NRBC is also present (Wright-Giemsa stain; 1000× magnification).

The other genotypes, β^0/β^+ and β^+/β^+, show some HbA, but the majority of the hemoglobin is HbF with normal to increased HbA_2[33] (Figure 14-10 ■). The increased HbF in thalassemia is thought to be due to the expansion of a subpopulation of erythrocytes that have the ability to synthesize γ-chains. The distribution of HbF among erythrocytes is heterogeneous.

Definitive diagnosis of β-thalassemia can be made by demonstration of a β-to-α-chain ratio of <0.25. Molecular techniques demonstrating specific genetic mutations can also define the presence of thalassemia (Chapter 42).

Treatment

Most children with β-thalassemia major participate in a regular transfusion program, which prolongs life into at least the second or third decade and allows normal development and growth patterns. Initial treatment protocols were mainly palliative and designed to maintain

	CA	CS	COE A2	GDS Lepore	F	A	Portland	Bart's	H
β-thal major	|		|		▮			
β-thal major (med)	|		|		▮	|		
β-thal intermediate	|		|		▮	▮		
β-thal minor	|		|		|	▮		
β-thal minima	|		|			▮		

CA = Carbonic anhydrase
CS = Constant Spring
med = Mediterranean

FIGURE 14-10 Hemoglobin electrophoresis pattern from patients with β-thalassemia shows a reduction in β-containing HbA and an increase in non-β-containing HbA_2 and HbF.

the hemoglobin level of approximately 7–8 g/dL (70–80 g/L), but evidence of erythroid expansion and increased iron absorption persisted. Clinical evidence suggests that the more aggressive hypertransfusion programs (maintaining a hemoglobin level of 9–10.5 g/dL) offer the highest quality of life without significant sequelae.[34] The objectives of hypertransfusion programs are to minimize the anemia, reduce excess iron absorption, and suppress ineffective erythropoiesis. The large doses of iron received with these transfusions, however, lead to tissue damage from iron overload similar to that seen in hereditary hemochromatosis. Iron-chelating agents such as deferoxamine and deferasirox are given to decrease the deposition of iron in the tissues.[35,36] Splenectomy can be performed in an attempt to decrease hemolysis and prolong red cell survival. However, splenectomy is usually reserved for patients older than 5 years of age who receive >200 mLs of packed red blood cells (PRBCs)/kg/yr, have leukopenia, thrombocytopenia, splenomegaly pain, or iron overload with chelation therapy.[35] Risks associated with splenectomy include sepsis, thrombosis, and possibly pulmonary hypertension.[37,38]

Bone marrow transplants (BMT) have been attempted in an effort to provide the individual with stem cells capable of producing normal erythrocytes. Although BMT is an option for thalassemia patients with a suitable donor, this technology is not widely used.[39] BMT can be replaced with stem cell transplants (SCT) that yield <10% mortality, minimal morbidity, and less infertility issues.[40] Clinical efficacy is controversial with the highest risk factor being a lack of engraftment. Event-free survival is <70% for BMT and between 80 and 90% for SCT in patients with β-thalassemia who receive HLA-identical, related BMT.[40–44] The success rate is lower for adults with advanced disease.[45] It is difficult to find HLA-matched related donors, and success is much lower (ranging from 20–76%) using matched, unrelated donors.[46–49] Haploidentical mother-to-child transplantation can be an option for children without a matched donor.[45] Cord blood transplants are being investigated and show promise.[50–52] With a typical life expectancy of around 50 years when patients follow recommended transfusion and chelation protocols, supportive therapy must be considered over curative therapy unless the patient is young, fit, and considered for transplant prior to developing thalassemia-related sequalae.[4]

Drugs to treat leukemia are being used to induce the re-expression of latent γ-genes that would combine with the excess α-chains and produce HbF.[53–55] The two major risk factors for this treatment approach include failure of γ-chain stimulation and induction of malignancy from the antileukemic drugs.[56,57]

There is continuing interest in gene therapy approaches in which autologous stem cells are harvested and the β-gene complex transfected using viral vectors and transplanted back into the host. However, several complications prevent widespread use. Human globin genes are large and complicated, making the finding of adequate viral vectors difficult. In clinical trials of gene therapy for severe immunodeficiency diseases, some patients have developed leukemias when the transfected genes insert next to or within critical hematopoiesis control genes.[58–60] The first thalassemia patient to receive gene therapy was reported in 2007.[61]

The use of induced pluripotent stem cells (iPS) shows promise for hematopoietic and genetic disorders in the future. Somatic skin fibroblasts can be harvested and reprogrammed into iPSs by inducing the expression of Oct4, Sox2, Klf4, and c-Myc, transcription factors essential for maintaining pluripotency in early embryos and embryonic stem cells.[62,63] When grown in culture, these cells could be induced to differentiate into multipotent hematopoietic stem cells and then autotransplanted.

Although significant obstacles must be overcome, correction of the molecular defect with gene therapy is thought to be achievable in the future.[64]

Prognosis

Untreated patients generally expire during the first or second decade of life. Patients enrolled in a hypertransfusion program with chelation therapy can extend their life expectancy by at least a decade.[65] Usually in the second decade of life, endocrine disorders (e.g., diabetes) and hepatic and cardiac disturbances develop from excessive deposits of iron in these tissues if chelation therapy has not been successful.

β-Thalassemia Minor (β^0/β or β^+/β)

Expected Genotypes

β-Thalassemia minor results from the heterozygous inheritance of either a β^+- or β^0-gene with one normal β-gene (Table 14-5). No major clinical difference seems to exist in the expression between the two thalassemia genes in the heterozygous state. About 1% of African Americans are heterozygous for β-thalassemia.

Pathophysiology

The normal β-gene directs synthesis of sufficient amounts of β-chains to synthesize enough HbA for nearly normal oxygen delivery and erythrocyte survival. In the case of a heterozygous β^+ patient, the thalassemic gene will also contribute to β-chain production.

Clinical Findings

The heterozygote appears to be asymptomatic except in periods of stress that can occur during pregnancy and with infections. Under such conditions, a moderate microcytic anemia can develop. Concomitant folate deficiency can produce a macrocytic anemia.

Laboratory Results

The anemia in β-thalassemia minor is mild with hemoglobin values in the range of 9–14 g/dL with the mean value for women being 10.9 g/dL and for men 12.9 g/dL.[66–68] The erythrocyte count is increased (>5 $\times$ 10^{12}/L) for what is expected at the given hemoglobin concentration. The condition is usually discovered incidentally during testing for unrelated symptoms or during family study workups.

The erythrocytes are microcytic (MCV = 55−70 fL) and hypochromic (MCHC = 29−33 g/dL) or sometimes normochromic with an MCH that is usually <22 pg (Figure 14-10). The degree of microcytosis, as indicated by the MCV, is directly related to the severity of the anemia.[30] Although the anemia is mild, the peripheral blood smear shows variable anisocytosis and poikilocytosis with target cells and basophilic stippling. Nucleated red blood cells are not usually found, but anemic patients can have a slightly elevated reticulocyte count. Bone marrow shows slight erythroid hyperplasia and erythroblasts poorly filled with hemoglobin.

Hemoglobin electrophoresis demonstrates an increase in HbA_2 of 3.5–7.0% with a mean of 5.5%. Newborns have a normal HbA_2 concentration of 0.27 ± 0.02%.[32] HbF is normal in approximately half of the patients and increased in the other half (Figure 14-10).[69,70] If HbF exceeds 5%, however, the individual has probably inherited an HPFH gene (see "Hereditary Persistance of Fetal Hemoglobin") in addition to the β-thalassemia gene. Vital stains to detect Heinz bodies are usually negative. In summary, β-thalassemia minor is indicated when the MCH is <27 pg and the HbA_2 is >3.5%.

Nucleic acid–based techniques can be performed to identify the type of mutation present and validate the heterozygous inheritance pattern but are of limited diagnostic value. Such information, however, can be helpful in counseling prospective parents with β-thalassemia minor.

☑ CHECKPOINT 14-7

In β-thalassemia, what erythrocyte parameter on the CBC differs significantly from that found in iron deficiency?

Treatment and Prognosis

Patients generally do not require treatment if they maintain good health and nutrition. They are generally asymptomatic except during periods of physiologic stress, and they have a normal life expectancy.

β-Thalassemia Intermedia (β^+/β^+, β^0/β^+, β^0/β)

Expected Genotypes

All three patterns of inheritance—homozygous, double heterozygous, and heterozygous—can produce β-thalassemia intermedia (Table 14-5). The homozygous and double heterozygous forms represent a mutation in both β-alleles, resulting in a moderate reduction in β-chain synthesis. Patients who inherit a mutation of one β-gene in conjunction with a normal β-gene occasionally exhibit clinical symptoms significant enough to be classified as β-thalassemia intermedia rather than β-thalassemia minor.

Patients with β-thalassemia who co-inherit α-thalassemia or HPFH can actually experience milder symptoms as compared with those with pure β-thalassemia. In both cases, excess free α-chain accumulation and precipitation are reduced, decreasing the ineffective erythropoiesis and extravascular hemolysis responsible for much of the pathology. In the case of co-inherited β-thalassemia and HPFH, the overexpressed γ-chains combine with the excessive α-chains to produce HbF, which reduces α-chain precipitation.

Clinical Findings

Patients with β-thalassemia intermedia present with symptoms of intermediate clinical severity between severe β-thalassemia major and mild β-thalassemia minor. The β^0/β^+-genotype produces the greatest reduction in β-chain synthesis but has a variable clinical presentation; some patients have symptoms of β-thalassemia major, whereas others have a much milder clinical phenotype. Patients who inherit the β^0/β-genotype generally exhibit mild symptoms. The criterion for β-thalassemia intermedia is the ability to maintain a hemoglobin level associated with a comfortable survival without requiring regular transfusions.[34] The need for transfusions is defined by the quality of life, not the hemoglobin level per se. Some patients with a hemoglobin level of 7 g/dL can be relatively symptom free, but others with a hemoglobin of 9 g/dL can have clinical symptoms related to ineffective erythropoiesis. Clinical symptoms often intensify during periods of physiological stress as with pregnancy and infection and can require short-term transfusion therapy. Even in the absence of regular transfusions, some patients develop progressive iron overload and require iron-chelation therapy.

Laboratory Results

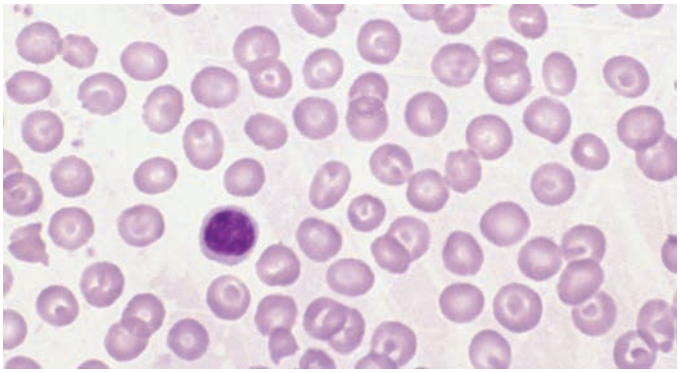
The CBC reflects a moderate microcytic hypochromic anemia with a hemoglobin value range of 7–10 g/dL (Figure 14-11 ■). In milder cases, patients express only a slight reduction in hemoglobin values. The erythrocyte count disproportionately higher than the hemoglobin values and often approaches normal.

Target cells are the predominant poikilocytes observed. Basophilic stippling and nucleated red blood cells are also present. The bone marrow shows hypochromic erythroblasts in the context of erythroid hyperplasia. However, bone marrow examination is not needed for diagnosis.

Hemoglobin electrophoresis patterns in patients with the more severe forms of β-thalassemia intermedia ($\beta^0\beta^+$ and $\beta^+\beta^+$) are nearly indistinguishable from those observed in the milder forms of β-thalassemia major. Patients express elevated HbA_2 (5–10%) and HbF (30–75%) with the remainder being HbA. Milder versions of β-thalassemia intermedia produce lower HbA_2 (>3.2%) and HbF levels (1.5–12.0%) (Figure 14-10). Although hemoglobin electrophoresis is helpful in diagnosing β-thalassemia intermedia, differentiation from β-thalassemia major and minor is a clinical decision.

Treatment and Prognosis

Splenomegaly is common. Functional hyposplenism leads to infections requiring regular interventions with antibiotic therapy. Chelation therapy may be warranted to combat iron overload, which tends



■ **FIGURE 14-11** Patients with β-thalassemia minor show minimal morphologic abnormalities to include microcytosis with target cells. The CBC in this patient showed the following results: Hb 11.1 g/dL; RBC count 5.2 × 10^{12}/L; MCV 61 fL; MCH 20.2 pg; MCHC 33 g/L (Wright-Giemsa stain; 1000× magnification).

to develop later than in patients with β-thalassemia major. Most patients have a normal life span.

β-Thalassemia Minima (β^{SC}/β)

β-Thalassemia minima is a form of asymptomatic β-thalassemia, exhibits no major laboratory abnormalities, and is only defined by a mildly imbalanced α- to non-α globin chain synthesis ratio. The disorder is usually discovered serendipitously during family studies. The gene has been given the designation β^{SC} for silent carrier. The genotype used to describe a patient with β-thalassemia minima is β^{SC}/β.[71] Homozygosity for β^{SC} or combination of the silent allele with other β-thal genes results in a mild form of thalassemia intermedia.

CASE STUDY *(continued from 261)*

Additional tests were performed on John's blood to determine the cause of his anemia.

Hemoglobin	Electrophoresis	Iron Panel	
HbA	66%	Serum iron	92 mcg/dL
HbA_2	1%	TIBC	310 mcg/dL
HbF	1%	Serum ferritin	88 ng/mL
Hb Bart's	8%	Iron saturation	33%
HbH	24%		

5. Is the hemoglobin electrophoresis normal or abnormal?
6. If abnormal, list hemoglobins that are elevated, decreased, or abnormally present.
7. If abnormal, which globin chain(s) is (are) decreased?
8. If abnormal, which globin chains are produced in excess?
9. Is the iron panel normal or abnormal?
10. If the iron tests are abnormal, list those outside the reference interval and indicate whether they are elevated or decreased.
11. If the iron tests are abnormal, state the disorder(s) consistent with the abnormal iron panel.
12. Given all the data supplied, what is the definitive diagnosis of John's anemia?

OTHER THALASSEMIAS AND THALASSEMIA-LIKE CONDITIONS

In theory, any globin gene can be mutated, resulting in a reduction in the synthesis of globin chains and the corresponding hemoglobin. Individuals have been observed with deficiencies in each of the normal adult globin chains. However, the thalassemias that involve globin chains other than α and β are relatively benign in their clinical course because they are not constituents of the major adult hemoglobin, HbA. Thalassemias have been observed involving more than one globin gene and in combination with structural hemoglobin disorders such as sickle cell anemia and HbC disease (Table 14-6 ★).

δβ-Thalassemia

δ-, β-thalassemia (δβ-thalassemia) is a rare thalassemia observed primarily in patients of Greek, African, Italian, and Arabian ancestry whose production of both β- and δ-chains is affected. The δβ-mutation can be categorized into two genotypes, $\delta\beta^0$ and $\delta\beta^+$. The $\delta\beta^0$ designation indicates a complete lack of synthesis of both β- and δ-chains from a given chromosome, whereas the $\delta\beta^+$-genotype indicates a reduction in β- and δ-chain synthesis. The absence of β- and δ-chains is most often due to deletion of the structural β- and δ-gene complex. One or both of the γ-genes remain, resulting in 100% HbF in homozygous $\delta\beta^0/\delta\beta^0$.[66] However, increased γ-chain production fails to fully compensate for the loss of β-chain production, resulting in a thalassemic phenotype and anemia.

In δβ-thalassemia, the γ-chain synthesis is less than in HPFH but more than in homozygous β^0-thalassemia. Clinically, the disease is classified as thalassemia intermedia and rarely requires blood transfusions except in cases of physiological stress such as pregnancy or infection. However, because of the impaired hemoglobin synthesis, most patients with δβ-thalassemia have a mild hypochromic, microcytic anemia. Patients have slight hepatosplenomegaly and some bone changes associated with chronic erythroid hyperplasia. Hemolysis probably contributes to the anemia because both reticulocytes and bilirubin are elevated.

The heterozygous form of δβ-thalassemia ($\delta\beta^0/\beta$) is not identified with any specific clinical finding. There is no anemia or splenomegaly. The hematological picture, however, is similar to that of β-thalassemia minor with microcytic, hypochromic erythrocytes. HbA_2 is normal or slightly decreased, whereas HbF is increased to 5–20%. HbA is usually <90% (Table 14-6).

γδβ-Thalassemia

This rare form of thalassemia (γ-, δ-, β-thalassemia has several variants and is characterized by deletion or inactivation of the entire β-gene complex.[33] Deletion of the γ-, δ-, and β-genes would result in the absence of all normal adult hemoglobin production from that chromosome. Therefore, only the heterozygous state has been encountered because a homozygous condition would be incompatible with life. Although neonates have severe hemolytic anemia, as the children grow, the disease evolves to a mild form of β-thalassemia.

Hemoglobin Constant Spring

Hemoglobin Constant Spring (HbCS) is a hemoglobin tetramer formed from the combination of two structurally abnormal α-chains, each elongated by 31 amino acids at the carboxy-terminal end, and two normal β-chains. This genetic mutation is common in Thailand. The chromosome with the α^{CS} gene carries one normal α-gene. Thus, the homozygous HbCS individual has two normal α-genes, one on each chromosome, and the heterozygous HbCS carrier has three normal α-genes.

The elongated α-chains of HbCS are thought to result from a mutation of the α-chain termination codon by a single base substitution.[72] The abnormal α-chains are synthesized at very low levels (~1%

★ **TABLE 14-6** Characteristics of Other Thalassemias and Thalassemia-Like Conditions

Disorder	Defect	Ancestry	CBC/diff	Hb Electrophoresis	Other
$\delta\beta$-thalassemia homozygous ($\delta\beta^0/\delta\beta^0$)	Deletion of $\delta\beta$-gene complex	Greek, African, Italian, Arabian	Micro/hypo RBCs Hb 10–12 g/dL	↓↓ HbA, ↓↓HbA_2, ↑↑ HbF	Thalassemia intermedia
heterozygous ($\delta\beta^0/\beta$)		Same as homozygous	No anemia	↓ HbA, N-↓HbA_2, ↑ HbF	Thalassemia minor
$\gamma\delta\beta$-thalassemia heterozygous ($\gamma\delta\beta^0/\beta$)	Deletion or inactivation of β-gene complex	Mediterranean regions	Birth: Marked anemia; Adult: Slight anemia	↓HbA, ↓HbA_2, ↓ HbF	Thalassemia minor
homozygous ($\gamma\delta\beta^0/\gamma\delta\beta^0$)		Same as heterozygous	Unable to observe	No adult Hb	Incompatible with life
Constant Spring homozygous ($\alpha\alpha^{CS}/\alpha\alpha^{CS}$)	Long α-chain, mutated stop codon, reduced synthesis	Thailand	Micro/hypo RBCs, Hb 9–11 g/dL, ↑ reticulocytes	Hb Bart's at birth, ↑↑ HbCS, ↓HbA, N-HbA_2 and HbF	
heterozygous ($\alpha\alpha^{CS}/\alpha\alpha$)		Same as homozygous	Normal	↑ HbCS	Can be seen with α-thal2 trait
HPFH homozygous ($\delta\beta^0/\delta\beta^0$)	Deletion or inactivation of $\delta\beta$-gene complex	Greek, Swiss, Black	Mild micro/hypo RBCs, no anemia, ↑↑RBCs, Hb 14–18 g/dL	100% HbF	Two variants—pancellular, heterocellular Asymptomatic
heterozygous ($\delta\beta^0/\beta$)		Same as homozygous	Near normal	↑ HbF, ↓ HbA, ↓ HbA_2	Asymptomatic
Hb Lepore homozygous ($\delta\beta^{Lepore}/\delta\beta^{Lepore}$)	$\delta\beta$-hybrid chain	European	Marked micro/hypo RBCs, Hb 4–11 g/dL, ↑↑↑ anisopoikilocytosis	No HbA, No HbA_2, ↑↑ Hb Lepore, ↑↑ HbF	Thalassemia major or intermedia
heterozygous ($\delta\beta^{Lepore}/\beta$)		Same as homozygous	Slight micro/hypo RBCs, Hb 12 g/dL	↓ HbA, ↓ HbA_2 ↑ Hb Lepore	Thalassemia minor

↑↑↑ = marked increase; ↑↑ = moderate increase; ↑ = slight increase; ↓↓ = moderate decrease; ↓ = slight decrease; N = normal

of the output compared with a normal α gene) because of reduced stability of the elongated mRNA. The result is an overall deficiency of α-chain synthesis producing an α-thalassemia-like phenotype.

Clinical Findings

The homozygous state is phenotypically similar to α-thalassemia minor. A slight anemia accompanied by mild jaundice and splenomegaly is typical. Heterozygotes show no clinical abnormalities.

Laboratory Results

In homozygotes, clinical findings are similar to a relatively mild form of HbH disease with a mild microcytic hypochromic anemia. Hemoglobin electrophoresis demonstrates the presence of Hb Bart's at birth. In homozygous adults, HbCS makes up 5–7% of the hemoglobin, and HbA_2 and HbF are normal. The remainder of hemoglobin is HbA (Figure 14-2).

Heterozygotes show no hematological abnormalities, but a small amount of HbCS (0.2–1.7%) can be found on electrophoresis. HbA_2 and HbF are normal with the remainder being HbA (Figure 14-2).

In some areas, the coexistence of HbCS with α-thalassemia trait ($--/\alpha\alpha^{CS}$) can be found. The clinical findings are similar to those of HbH disease. Hemoglobin electrophoresis characteristically shows HbA, HbH, Hb Bart's, HbA_2, and 1.5–2.5% HbCS[73] (Figure 14-2; Table 14-6).

☑ CHECKPOINT 14-8

Why is $\gamma\delta\beta$-thalassemia more severe than $\delta\beta$-thalassemia and CS-thalassemia?

Hereditary Persistence of Fetal Hemoglobin (HPFH)

Hereditary persistence of fetal hemoglobin (HPFH) is actually a group of heterogeneous disorders caused by either the absence of δ- and β-chain synthesis or a loss of suppression of the γ-globin gene, resulting in an increased γ-chain production into adult life. In homozygotes, the result is an absence of HbA and HbA_2 with 100% of hemoglobin production being HbF. Hemoglobin F production continues at high levels throughout life, preventing the clinical symptoms and hematological abnormalities associated with thalassemia. The condition occurs in 0.1% of African Americans.

Genetics

HPFH is characterized by either deletion or inactivation of the β- and δ-structural gene complex, mutations in the γ-globin gene promoter region affecting the binding of transcription factors,[74–76] or mutation of gene inhibitor proteins.[77–81] Four potential mechanisms that

explain the increase in γ-globin gene expression have been reported. Removal of the δ and β promoters: (1) relieves competition with the γ-gene promoters for transcription factor activation or (2) removes the γ-gene inhibitor–binding sites; (3) mutations might juxtapose β-gene enhancer sequences to γ-genes, facilitating activation of γ-genes; and (4) mutated transcription factors increase activation or inhibit suppression of the γ-globin gene.[4] Most α-chains combine with the available γ-chains to produce HbF. Consequently, no accumulation and no precipitation of excess α-chains occur. In HPFH, increased production of γ-chains with the corresponding elevations of HbF compensates for the reduction in HbA and HbA_2 synthesis and differentiates it from δβ-thalassemia in which only modest elevations in HbF are observed.

Variants

HPFH can be categorized into two major groups, pancellular and heterocellular, based on the distribution of HbF in erythrocytes. The HbF distribution patterns can be visualized using the acid elution stain developed by Kleihauer and Betke. *Pancellular* refers to the observance of HbF in most of the erythrocytes; in the *heterocellular* version, HbF is concentrated in a small subset of erythrocytes.

Several different types of HPFH have been described: the Black, Greek, and Swiss types. In the Black and Swiss types, both $^{G}\gamma$- and $^{A}\gamma$-chains are produced in approximately equal amounts. The Greek form is characterized by production of both $^{G}\gamma$- and $^{A}\gamma$-chains, but most HbF is made up of the $^{A}\gamma$-chains. Both the Black and Greek types have the characteristic pancellular distribution pattern of HbF in erythrocytes. The Swiss form exhibits a heterocellular distribution of HbF, which results from a hereditary increase (3%) in the number of fetal (F) cells[21,22] (Table 14-7 ★).

When present, the pancellular distribution of HbF in erythrocytes helps to distinguish this disorder from other disorders associated with an increase in HbF. Most other disorders with elevated HbF levels present with the heterocellular distribution pattern.

It has been suggested that the various categories of HPFH actually represent a continuum of a spectrum of β-thalassemias with homozygous β^0-thalassemia at one end where the lack of β-chain synthesis is poorly compensated for by γ-chain production and with pancellular HPFH at the other end where the lack of β-chain synthesis is almost completely compensated for by γ-chain production.

Homozygotes

Homozygous HPFH is asymptomatic, including no evidence of abnormal growth patterns or splenomegaly. The abundant γ-chains combine with the normal α-chains to produce HbF.

★ **TABLE 14-7** Characteristics of HPFH Variants

HPFH Type	Types of γ-chains Produced	Distribution of HbF in Erythrocytes
Black	$^{G}\gamma$- and $^{A}\gamma$-	Pancellular
Swiss	$^{G}\gamma$- and $^{A}\gamma$-	Heterocellular
Greek	Primarily $^{A}\gamma$-	Pancellular

Erythrocytosis ranging from 6×10^{12}/L to 7×10^{12}/L occurs as the result of the higher oxygen affinity of HbF as compared with to HbA. Corresponding high hemoglobin levels from 14.8–18.2 g/dL are also typical in HPFH. Erythrocytes are microcytic and slightly hypochromic with a mean MCV of 75 fL and a mean MCH of 25.0 pg. There is a mild degree of anisocytosis and poikilocytosis. The reticulocyte count range is 1–2%. It is doubtful that this disorder has any significant degree of hemolysis because the reticulocyte count, bilirubin, and haptoglobin levels are normal. Electrophoresis demonstrates 100% HbF.

Heterozygotes

Heterozygous HPFH is usually found incidentally through family studies. Patients present with a slightly elevated erythrocyte count with the corresponding elevation of the hematocrit and a slightly decreased MCH (27 pg). HbF is 10–30% of the total hemoglobin. HbA_2 is decreased to 1–2% and the remainder is HbA. In the presence of iron deficiency, HbF levels are lower (Table 14-6).

Hemoglobin Lepore

Incidence and Affected Individuals

Hemoglobin Lepore was first described in 1958 as a structural hemoglobin variant with hematological changes and clinical manifestations resembling those of thalassemia.[73,82] The disorder is widely distributed throughout the world but is especially common in middle and eastern Europe.

Genetics

In Hb Lepore, the non-α-chain is a δ/β-globin hybrid in which the N-terminal end of a δ-chain is fused to the C-terminal end of a β-chain. The variant hybrid genes are thought to occur during meiosis from an aberrant **crossover** event resulting in recombination of misaligned δ- and β-genes on separate chromosomes. The result of the unequal crossover event is two fusion genes, the δ/β-Lepore and the β/δ-anti-Lepore genes. The δ/β-Lepore fusion gene is transcribed and translated into the δ/β-fusion globin chain, two of which combine with two α-chains to form Hb Lepore. The chromosome containing the β/δ-anti-Lepore fusion gene still contains intact β- and δ-genes that are synthesized normally to form HbA and HbA_2, respectively. Progeny bear genes from the involved chromosome that are neither fully paternal nor fully maternal. Because the recombination event occurred in the germ cells, the newly formed chromosomes become a permanent part of the family's gene pool. Hb Lepore is stable and has normal functional properties except for a slight increase in oxygen affinity.

Pathophysiology

The pathophysiology of hemoglobin Lepore is similar to that of β-thalassemia. No intact β-gene is present on the chromosome carrying the Lepore fusion gene, so β-chain synthesis is absent. The Hb Lepore gene is under the influence of the δ-gene promoter, which limits synthesis of the Lepore hybrid chain to approximately 2.5% of normal β-chain production. Thus, the abnormal Lepore chains are inadequately synthesized, leading to an excess of α-chains. In the homozygous state, no normal β-chains or δ-chains would be

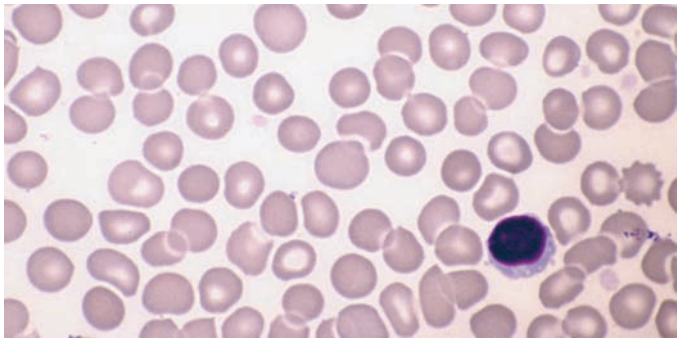


FIGURE 14-12 Peripheral blood smear from a patient with hemoglobin Lepore. Note the anisocytosis, poikilocytosis, and microcytosis (Wright-Giemsa stain; 1000× magnification).

synthesized to combine with the α-chains being produced, thus, no HbA or HbA_2. The more severely affected children can become transfusion dependent and develop complications of hemosiderosis. The combination of ineffective erythropoiesis, decreased HbA, increased oxygen affinity of HbF, and chronic extravascular hemolysis produces a microcytic hypochromic anemia that is classified clinically as β-thalassemia major in the more severe cases and as β-thalassemia intermedia in the remaining cases. As with β-thalassemia major, symptoms emerge within the first few years of life.

Patients with heterozygous Hb Lepore are asymptomatic and classified clinically as β-thalassemia minor. The blood picture is similar to that seen in β-thalassemia minor.

Laboratory Results

In the homozygous state, hematologic findings are similar to those of β-thalassemia major. There is no detectable HbA or A_2 on hemoglobin electrophoresis, Hb Lepore ranges from 8–30%, and the remainder consists of HbF. Hemoglobin electrophoresis must be interpreted with caution because Hb Lepore comigrates with HbS on cellulose acetate and agarose at an alkaline pH and with HbA on citrate agar at an acid pH.

In the heterozygous state, hematologic findings are similar to thalassemia minor (Figure 14-12 ■): Hemoglobin electrophoresis reveals a mean Hb Lepore concentration of 10%; HbA_2 is decreased with a mean of 2%; HbF is usually slightly elevated to 2–3%; and HbA makes up the remainder (Table 14-6).

The severely anemic cases of Hb Lepore require a regular transfusion protocol from early childhood. Splenectomy can be performed in an attempt to lessen the degree of anemia.

Combination Disorders

Occasionally an individual is doubly heterozygous for a structural hemoglobin variant and thalassemia, inheriting one of each of the two abnormalities from each parent. The most common structural hemoglobin variants involved in combination disorders are HbS, HbC, and HbE. When a structural variant is inherited with a β-thalassemia gene, the severity of the combination disorder depends on the type of β-gene mutation. Patients expressing the β^0-gene produce no HbA and experience moderate to severe symptoms. The β^+-gene produces some β-chains, resulting in HbA synthesis and few to no thalassemia symptoms. The most common example is HbS/β-thalassemia accounting for 1 in 1667 births among African Americans.[83] This has also been reported in patients of Greek, Turkish, Indian, North American, Mediterranean, and Romanian ancestry.[84] Three clinical severities have been identified: HbS/β^0-Type 1 (severe), HbS/β^+-Type 1 (moderate), and HbS/β^+-Type 2 (asymptomatic). Differentiating this combination disorder from sickle cell disease (HbSS) and trait (HbAS) is sometimes difficult, but a comparison of β:α ratio can be helpful (Web Table 14-1). In sickle cell disorders, the β:α ratio is approximately 1:1, whereas it is closer to 0.5:1 in HbS/β-thalassemia.[85]

Combination disorders are more complex when the structural hemoglobin gene mutation is on the β-gene and the thalassemia mutation involves the α-gene because different chromosomes are involved. These patients can be either homozygous or heterozygous for the structural hemoglobin variant and coexpress any of the possible α-gene combinations.

The severity of the combination disorder is directly proportional to the total number of affected genes and ranges from moderate to asymptomatic.[86–88] Coexistent α-thalassemia decreases synthesis of α-chains resulting in fewer α-globin chains available to combine with the structurally abnormal β-chain. Thus, the concentration of the structural variant (in heterozygotes) is usually decreased because the limited number of α-chains preferentially combines with the normal β- or δ- (or γ-) chains. The percentage of HbA, HbF, and HbA_2 increase relative to a typical heterozygote, reducing the abnormal pathophysiology associated with the particular structural hemoglobin variant inherited.

In the case of a homozygous $\beta^S\beta^S$ individual with an α-thalassemia syndrome, the clinical severity of sickling is often reduced because of a net decrease in MCHC, which reduces the tendency of HbS to produce cell sickling. Thus, cell hemolysis and the clinical symptoms associated with occlusion of the microvasculature decrease (Chapter 13). HbF has also been shown to decrease the sickling process.[13] The wide variety of clinical severities seen in sickle cell anemia can be partially related to the high incidence of α- and β-thalassemia in the same population (Figure 14-13 ■).

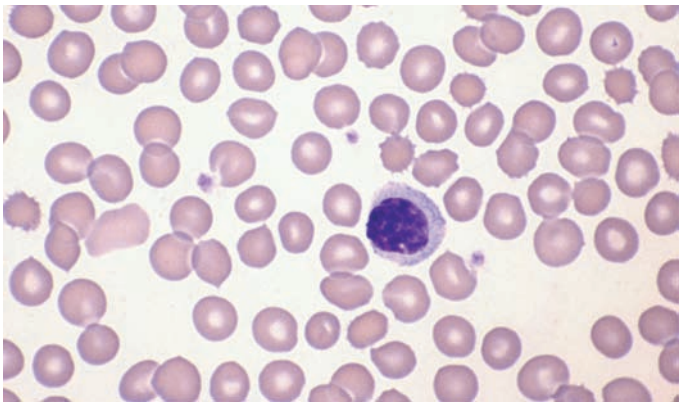


FIGURE 14-13 Peripheral blood smear from a patient with hemoglobin S and α-thalassemia. The cells are microcytic. There are acanthocytes and cells with pointed ends present (Wright-Giemsa stain; 1000× magnification).

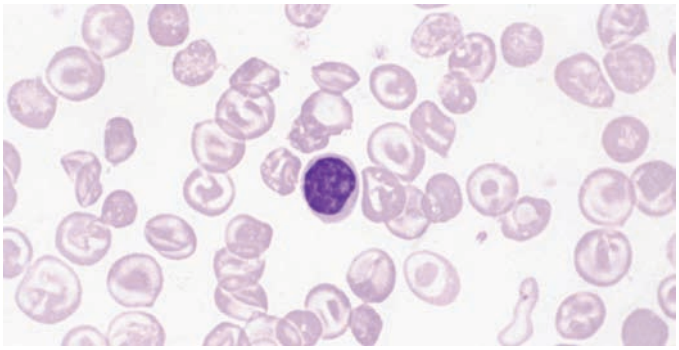
Laboratory diagnosis is accomplished by applying the techniques used to identify each of the disorders individually. Patients present with a mild microcytic, hypochromic anemia with target cells and the poikilocytes associated with the inherited structural hemoglobin variant. Patients who inherit HbS can have sickle cells, and those with HbC sometimes show HbC crystals. Hemoglobin electrophoresis is helpful in resolving the structural hemoglobin variant and shows the quantitative changes in normal and abnormal hemoglobins associated with α- and β-thalassemias. In HbS-α-thalassemia, the concentration of HbS is inversely proportional to the number of α-gene deletions. HbS concentration in heterozygotes is ~35% with one α-gene deletion, ~28% with two α-deletions, and ~20% with three α-gene deletions.[89]

When less common structural variants (HbE, HbO, HbD, etc.) are coexpressed with thalassemias, more testing could be necessary because they comigrate with HbS or HbC on hemoglobin electrophoresis (Figure 14-2). Molecular techniques including automated sequencing, dot-blot analysis, or allele-specific amplification can be used in these cases (Chapter 43).[90]

The incidence of double heterozygotes expressing both HbE and β-thalassemia is increasing in Southeast Asia, and many patients are presenting with symptoms that rival β-thalassemia major in severity.[91] Approximately 3000 people with both mutations are born each year in Thailand, and many others are diagnosed in other parts of Southeast Asia, India, and Burma. In Thailand, Laos, and Cambodia, HbE penetrance is approximately 50%. HbE heterozygotes usually have relatively mild disease; therefore, HbE/β-thal double heterozygotes might be expected to have a moderately severe disease.[92] However, the HbE mutation at the 26th position of the β-globin gene activates a cryptic splice site at codon 25, resulting in significantly reduced β-chain production. Therefore, in double heterozygotes of HbE and β^0-thal, few to no β^E- or β^A-chains are produced. This results in the accumulation of α-chains that precipitate within the erythrocytes, producing hemolysis as in β-thalassemia major.[92,93] A more moderate anemia can result if the β^+-thalassemia gene is inherited with HbE. In this form of the disease, there are HbE, HbA, HbF, and HbA_2. The HbA, however, is less than would be expected in HbE trait, and the anemia is more severe. When the β^+-thalassemia gene is inherited with the β^E-gene, the result is microcytic, hypochromic anemia with significant poikilocytosis, and nucleated erythrocytes.

Hemoglobin E can also be found in combination with α-thalassemia (Figure 14-14 ■). This combination produces a more severe anemia than does HbE alone. The amount of HbE depends on whether the patient is heterozygous or homozygous for HbE and the α-thalassemia genotype inherited (Chapter 13).

Double heterozygotes for sickle cell and HPFH (deletion variant) exhibit a mild form of sickle cell trait with no occurrence of crises or anemia. It has been suggested that the surprisingly favorable clinical picture is related to the distribution of HbF in erythrocytes. The peripheral blood smear shows anisocytosis and target cells, and the sodium metabisulfite and solubility tests are positive. Hemoglobin electrophoresis produces a pattern that is easily confused with that of sickle cell anemia. Only HbS, HbF, and HbA_2 are present with HbF levels of 15–35%. HbA_2 is normal or reduced. Family studies are helpful in identifying the double heterozygous state.



■ **FIGURE 14-14** Peripheral blood smear from a patient with hemoglobin E and α-thalassemia. The RBCs are microcytic, hypochromic, and target cells are present (Wright-Giemsa stain; 1000× magnification).

Due to the significant variations in clinical expression of the various hemoglobin structural variants and β-thalassemia alleles, it is suggested that identification of these conditions should go beyond hematologic analysis and hemoglobin electrophoresis.[92] A diagnosis defined at the molecular level to identify the genetic mutation can lead to better clinical management of the disease. A summary of the differentiating characteristics of combination thalassemia and hemoglobinopathy disorders can be found in Web Table 14-1.

☑ CHECKPOINT 14-9

In combination disorders of structural Hb variants and thalassemia, why is α-thalassemia inherited with sickle cell trait less severe than β-thalassemia coexpressed with sickle cell trait?

DIFFERENTIAL DIAGNOSIS OF THALASSEMIA

Clinical signs, symptoms, and CBC results are strikingly similar in microcytic, hypochromic anemias regardless of the etiology of the anemia, making the clinical diagnosis difficult. Differentiating the various thalassemias is even more difficult because they are all inherited and occur in similar nationalities. Additional laboratory tests are therefore crucial in making the differential diagnosis.[94,95] Web Table 14-2 summarizes the tests used to differentiate thalassemia from other anemias.

☑ CHECKPOINT 14-10

Which laboratory tests should be done first to differentiate thalassemia and iron deficiency?

Summary

Thalassemias result from genetic defects that affect the production of globin chains. Any of the globin chains can be affected, but the most clinically significant are α- and β-chain defects. The clinical severity of the disease is related to the number of mutated genes and the type of genetic defect. The human diploid genome normally has four α-genes, so in α-thalassemia, from one to four of the α-genes can be deleted. If only one gene is affected, the condition is not clinically or hematologically apparent, but if two or three are affected, both clinical and hematological abnormalities of mild or moderate severity, respectively, occur. Deletion of all four α-genes is incompatible with life. There are two β-alleles and three β-thalassemia gene defects; one causes a complete absence of β-chain production (β^0-thalassemia), one causes variably decreased synthesis of β-chain production (β^+-thalassemia), and the third minimally affects β-chain production (silent carrier or β^{SC}). This results in seven potential β-thalassemia genotypes ($\beta^0\beta^0$, $\beta^0\beta^+$, $\beta^+\beta^+$, $\beta^0\beta$, $\beta^+\beta$, β^{SC}/β^{SC}, β^{SC}/β) that are categorized into four clinical severities (β-thalassemia major, intermedia, minor, and silent carrier) ranging from severe to asymptomatic.

The thalassemias generally produce a microcytic, hypochromic anemia with changes in the concentrations of HbF, HbA_2, and HbA. HbF and HbA_2 are elevated in β-thalassemia and decreased in α-thalassemia with decreased concentrations of HbA in both. In the more severe α-thalassemias, HbH (β_4) and Hb Bart's (γ_4) can be detected. Erythrocyte morphology is similar in both major forms of thalassemia and in iron-deficiency anemia; however, hemoglobin electrophoresis and iron studies assist in differentiating these two entities. Some structural hemoglobin variants are synthesized in decreased quantities (i.e., Hb Lepore, Hb Constant Spring, HbE) and have clinical and morphologic similarities to thalassemias. Molecular techniques (Chapter 43) are now available to identify the genetic mutation in the globin gene but are not always necessary for diagnostic purposes.

Current therapies are improving, and medical access in underdeveloped countries is expanding. As a result of these advances, the general health and quality of life are improving for patients with thalassemia.

Review Questions

Level I

1. The statement that best defines thalassemia is: (Objective 1)
   A. qualitative disorder of hemoglobin synthesis derived primarily from a genetic point mutation in one or more globin genes
   B. disorder of inappropriate iron metabolism due to abnormal transferrin
   C. quantitative disorder of hemoglobin synthesis resulting from deletional and nondeletional mutations of globin genes
   D. single amino acid substitution in a globin chain affecting the function of hemoglobin
2. Which of the following statements is *false* for a patient with thalassemia but *true* in certain hemoglobinopathies? (Objective 2)
   A. Abnormal hemoglobin will polymerize inside erythrocytes, altering red cell shape.
   B. Novel hemoglobins composed of abnormal combinations of normal globin chains can be detected on hemoglobin electrophoresis.
   C. Elevations in embryonic and fetal hemoglobins can be observed.
   D. The amino acid sequence of the globin chains of the abnormal hemoglobins is normal.
3. What is the typical morphologic classification of erythrocytes in thalassemia? (Objective 3)
   A. macrocytic, normochromic
   B. normocytic, normochromic
   C. microcytic, hyperchromic
   D. microcytic, hypochromic
4. Select the disorder that is an α-thalassemia. (Objectives 5a, 5b)
   A. HbH disease
   B. Cooley's anemia
   C. HPFH
   D. Hb Lepore
5. α-Thalassemia is characterized by: (Objective 5c)
   A. deletion of β-genes
   B. amino acid substitutions in the α-chain
   C. excess α-chain production
   D. deletion of α-genes
6. Which nationality is *most* likely to be affected by thalassemia? (Objectives 5b, 6a)
   A. Chinese
   B. South American Indians
   C. Southeast Asians
   D. Europeans

7. Which of the following laboratory results would be expected in a patient with α-thalassemia? (Objective 5e)
   A. MCH = 32 pg
   B. MCV = 70 fL
   C. stomatocytes
   D. increased HbA
8. The pathogenesis of β-thalassemia includes: (Objective 6b)
   A. decreased production of β-chains
   B. abnormal structure of α-chains
   C. bone marrow hypoproliferation
   D. decreased synthesis of erythropoietin
9. In β-thalassemia major, hemoglobin electrophoresis will show: (Objective 6d)
   A. reduced HbF
   B. reduced HbA_2
   C. reduced HbA
   D. increased HbH
10. Select the thalassemia type in which the patient survives and presents with an abnormal hemoglobin that is sensitive to oxidation and precipitates in red cells after incubation with brilliant cresyl blue. (Objective 6d)
    A. hydrops fetalis
    B. HbH disease
    C. β-thalassemia minor
    D. silent carrier

Level II

1. α-Thalassemia most commonly results from which of the following genetic lesions? (Objective 1)
   A. gene deletion
   B. promoter mutation
   C. termination codon mutation
   D. splice site mutation
2. Why is hydrops fetalis incompatible with life? (Objective 4b)
   A. Life cannot exist without HbA.
   B. Lack of embryonic hemoglobins precludes fetal development.
   C. All three normal adult hemoglobins contain α-chains.
   D. Fetal hemoglobin is essential to sustain life after birth.
3. Which pathophysiologic event is involved in the pathogenesis of HbH disease? (Objective 4b)
   A. HbH has a higher affinity for oxygen that hampers oxygen release.
   B. HbH is an embryonic hemoglobin that is not present at birth.
   C. HbH cannot bind and transport oxygen.
   D. Polymerization of HbH alters erythrocyte shape.
4. The genetic designation heterozygous α^0-thal-1/normal refers to: (Objective 4a)
   A. α-thalassemia minor
   B. Cooley's anemia
   C. HbH disease
   D. silent carrier
5. The single best laboratory test to distinguish β-thalassemia minor from α-thalassemia, iron-deficiency anemia, HPFH, and hemoglobinopathies is: (Objective 8)
   A. hemoglobin solubility
   B. serum iron
   C. Heinz body stain
   D. HbA_2 level
6. Hemoglobin Constant Spring can best be described as: (Objective 7c)
   A. deletion of three α-genes
   B. two normal β-chains and two elongated α-chains
   C. two normal α-chains and two β/γ-fusion chains
   D. continued synthesis of γ-chains throughout adult life
7. Select the statement that best describes hereditary persistence of fetal hemoglobin. (Objective 7d)
   A. The homozygous state is incompatible with life.
   B. HbF is elevated in adults.
   C. It results from the deletion of the γ-gene.
   D. It is a form of β-thalassemia.
8. A 4-year-old male patient has a microcytic, hypochromic anemia. Hemoglobin electrophoresis shows 46% HbS, 49% HbA, 3.5% HbA_2, 1.5% HbF. His parents have no symptoms of anemia. What are his parents' most likely phenotypes? (Objectives 5, 7)
   A. sickle cell trait and β-thalassemia major
   B. sickle cell anemia and α-thalassemia
   C. sickle cell anemia and heterozygous β-thalassemia
   D. sickle cell trait and normal

9. Which of the following combination disorders would exhibit more severe symptoms? (Objective 7f)

   A. HbS and β-thalassemia minor

   B. HbC trait and α-thalassemia minor

   C. HPFH and β-thalassemia minor

   D. HbS and HPFH

10. A 28-year-old female from Laos who had a hemoglobin of 11.2 g/dL was diagnosed with iron-deficiency anemia. She was given iron supplements. Her reticulocyte count increased from 4% to 5% after 6 days of treatment. Six months later, she returned for a follow-up CBC. Her hemoglobin was 11.5 g/dL, and the red cells were microcytic (75 fL), normochromic. What reflex test should be done? (Objective 8)

    A. hemoglobin electrophoresis

    B. serum iron

    C. bone marrow

    D. serum ferritin

Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

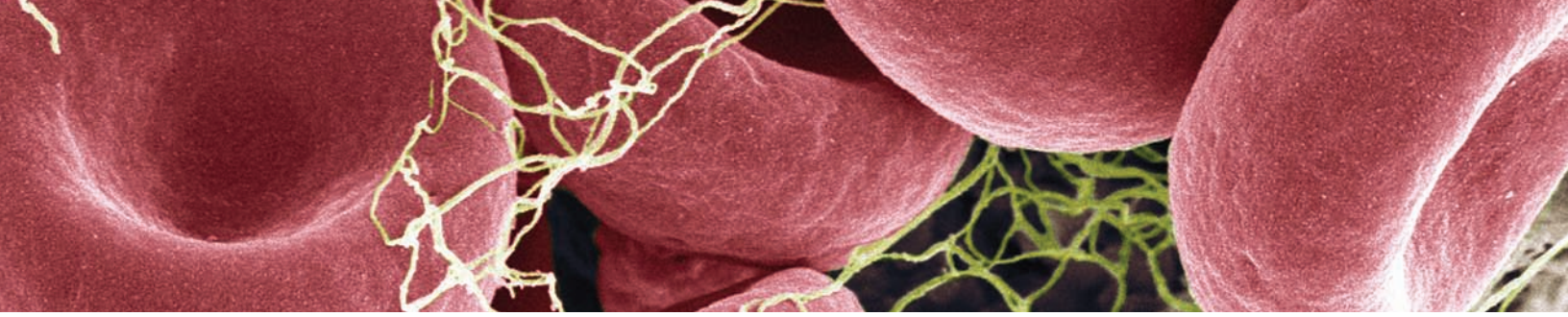
The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. HbVar: a database of human hemoglobin variants and thalassemias. http://globin.bx.psu.edu/cgi-bin/hbvar/counter; accessed March 15, 2012.
2. Cooley TB, Lee P. A series of cases of splenomegaly in children with anemia and peculiar bone changes. *Trans Am Pediatr Soc*. 1925;37:29.
3. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. *Bull World Health Organ*. 2008;86:480–87.
4. Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. *Lancet*. 2012;379(9813):373–83.
5. Sarnaik, SA. Thalassemia and related hemoglobinopathies. *Indian J Pediatr*. 2005;72:319–24.
6. Poole JH. Thalassemia and pregnancy. *J Perinat Neonatal Nurs*. 2003;17(3):196–208.
7. Mentzer WC. Differentiation of iron deficiency from thalassemia trait. *Lancet*. 1973;1(7808):882.
8. Muncie HL, Campbell JS. Alpha and beta thalassemia. *Am Fam Physician*. 2009;80(4):339–44, 371.
9. Cao A, Galanello R. Beta-thalassemia. *Genet Med*. 2010;12(2):61–76.
10. Old JM. Screening and genetic diagnosis of haemoglobin disorders. *Blood Review*. 2003;17:43–53.
11. Ristaldi MS, Pirastu M, Rosatelli C, Cao A. Prenatal diagnosis of β-thalassaemia in Mediterranean populations by dot blot analysis with DNA amplification and allele specific oligonucleotide probes. *Prenat Diag*. 1989;9:629–38.
12. Lo YM, Chiu RW. Noninvasive approaches to prenatal diagnosis of hemoglobinopathies using fetal DNA in maternal plasma. *Hematol Oncol Clin N Am*. 2010;24:1179–86.
13. Williams JL. Anemias of abnormal globin development. In: Steine-Martin EA, Lotspeich-Steininger CA, Koepke JA, eds. *Clinical Hematology: Principles, Procedures, Correlations*, 2nd ed. Philadelphia: Lippincott-Raven; 1998:217–40.
14. LeCrone CN, Detter JC. Screening for hemoglobinopathies and thalassemia. *J Med Technol*. 1985;2:389–95.
15. Harrison CR. Hemolytic anemias: intracorpuscular defects. In: Harmening DM, ed. *Clinical Hematology and Fundamentals of Hemostasis*, 3rd ed. Philadelphia: F.A. Davis; 1997:193–210.
16. Na-Nakorn S, Wasi P. Alpha-thalassemia in Northern Thailand. *Am J Hum Genet*. 1970;22:645–51.
17. Lehmann H, Carrell RW. Nomenclature of the alpha-thalassaemias. *Lancet*. 1984;1(8376):552–53.
18. Dozy AM, Kan YW, Embury SH et al. Alpha-globin gene organisation in blacks precludes the severe form of alpha-thalassemia. *Nature*. 1979;280:605–7.
19. Graham EA. The changing face of anemia in infancy. *Ped Rev*. 1994;15:175–83.
20. Huisman THJ, Carver MFH, Baysal E. *A syllabus of thalassemia mutations*. Augusta, GA: The Sickle Cell Anemia Foundation; 1997.
21. Giardina PJ, Rivella S. Thalassemia syndromes. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone; 2013:505–535.
22. Kattamis C, Hu H, Cheng G et al. Molecular characterization of beta-thalassaemia in 174 Greek patients with thalassaemia major. *Br J Haematol*. 1990;74:342–46.
23. Rachmilewitz E, Giardina PJ. How I treat thalassemia. *Blood*. 2011;118(13):3479–88.
24. Shinar E, Rachmilewitz EA, Lux SE. Differing erythrocyte membrane skeletal protein defects in alpha and beta thalassemia. *J Clin Invest*. 1989;83:404–10.
25. Advani R, Sorenson S, Shinar E et al. Characterization and comparison of the RBC membrane damage in severe human alpha and beta thalassemia. *Blood*. 1992;79:1058–63.
26. Yuan J, Kannan R, Shinar E et al. Isolation, characterization, and immunoprecipitation studies of immune complexes from membranes of [beta]-thalassemia erythrocytes. *Blood*. 1992;79:3007–13.
27. Mathias LA, Fisher TC, Zeng L et al. Ineffective erythropoiesis in beta-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. *Exp Hematol*. 2000;28:1343–53.
28. Schrier SL. Thalassemia: pathophysiology of red cell changes. *Ann Rev Med*. 1994;45:211–18.
29. Schrier SL. Pathophysiology of thalassemia. *Curr Opin Hematol*. 2002;9(2):123–26.
30. Rund D, Filon D, Strauss N et al. Mean corpuscular volume of heterozygotes for beta-thalassemia correlates with the severity of mutations. *Blood*. 1992;79:238–43.
31. Bagar MS, Khurshid M, Molla A. Does red blood cell distribution width (RDW) improve evaluation of microcytic anaemias? *JPMA J Pak Med Assoc*. 1993;43:149–51.
32. Steinberg MH, Coleman MB, Adams JG III. Beta-thalassemia with exceptionally high hemoglobin A2: differential expression of the delta-globin gene in the presence of beta-thalassemia. *J Lab Clin Med*. 1982;100:548–57.
33. Fearon ER, Kazazian HH Jr, Waber PG et al. The entire beta-globin gene cluster is deleted in a form of gamma delta beta-thalassemia. *Blood*. 1983;61:1269–74.

34. Forget BG, Cohen AR. Thalassemia syndromes. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Procedures*, 4th ed. Philadelphia: Churchill Livingstone; 2005:557–89.
35. Porter JB, Shah FT. Iron overload in thalassemia and related conditions: therapeutic goals and response to chelation therapies. *Hematol Oncol Clin N Am*. 2010;24:1109–30.
36. Porter JB. Deferasirox—current knowledge and future challenges. *Ann NY Acad Sci*. 2010;1202:87–93.
37. Phrommintikul A, Sukonthasarn A, Kanjanavanit R, Nawarawong W. Splenectomy: a strong risk factor for pulmonary hypertension in patients with thalassaemia. *Heart*. 2006;92:1467–72.
38. Singer ST, Kuypers FA, Styles L et al. Pulmonary hypertension in thalassemia: association with platelet activation and hypercoagulable state. *Am J Hematol*. 2006;81:670–75.
39. Lucarelli G, Weatherall DJ. For debate: bone marrow transplantation for severe thalassaemia. *Br J Haematol*. 1991;78:300–3.
40. Krishnamurti L, Bunn HF, Williams AM et al. Hematopoietic cell transplantation for hemoglobinopathies. *Curr Probl Pediatr Adolesc Health Care*. 2008;38:6–18.
41. Lawson SE, Roberts IA, Amrolia P et al. Bone marrow transplantation for β-thalassaemia major: the UK experience in two paediatric centres. *Br J Haematol*. 2003;120:289–95.
42. Sankaran VG, Nathan DG. Thalassemia: an overview of 50 years of clinical research. *Hematol Oncol Clin N Am*. 2010;24(6):1005–20.
43. Pinto FO, Roberts I. Cord blood stem cell transplantation for haemoglobinopathies. *Br J Haematol*. 2008;141:309–24.
44. Smiers FJ, Krishnamurti L, Lucarelli G. Hematopoietic stem cell transplantation for hemoglobinopathies: current practice and emerging trends. *Pediatr Clin North Am*. 2010;57:181–205.
45. Lucarelli G, Isgrò A, Sodani P, Gaziev J. Hematopoietic stem cell transplantation in thalassemia and sickle cell anemia. *Cold Spring Harb Persp Med*. 2012;2(5):a011825.
46. La Nasa G, Giardini C, Argiolu F et al. Unrelated donor bone marrow transplantation for thalassemia: the effect of extended haplotypes. *Blood*. 2002;99:4350–56.
47. La Nasa G, Caocci G, Argiolu F et al. Unrelated donor stem cell transplantation in adult patients with thalassemia. *Bone Marrow Transplant*. 2005;36:971–75.
48. Yannaki E, Emery DW, Stamatoyannopoulos G. Gene therapy for β-thalassaemia: the continuing challenge. *Expert Rev Mol Med*. 2010;12:e31.
49. Lucarelli G, Gaziev J. Advances in the allogeneic transplantation for thalassemia. *Blood Rev*. 2008;22:53–63.
50. Boncimino A, Bertaina A, Locatelli F. Cord blood transplantation in patients with hemoglobinopathies. *Transfus Apher Sci*. 2010;42:277–81.
51. Hall JG, Martin PL, Wood S, Kurtzberg J. Unrelated umbilical cord blood transplantation for an infant with β-thalassemia major. *J Pediatr Hematol Oncol*. 2004;26:382–85.
52. Jaing TH, Hung IJ, Yang CP et al. Rapid and complete donor chimerism after unrelated mismatched cord blood transplantation in 5 children with β-thalassemia major. *Biol Blood Marrow Transplant*. 2005;11:349–53.
53. Pace BS, Zein S. Understanding mechanisms of γ-globin gene regulation to develop strategies for pharmacological fetal hemoglobin induction. *Dev Dyn*. 2006;235;1727–37.
54. Perrine SP. Fetal globin stimulant therapies in the β-hemoglobinopathies: principles and current potential. *Pediatr Ann*. 2008;37:339–46.
55. Trompeter S, Roberts I. Haemoglobin F modulation in childhood sickle cell disease. *Br J Haematol*. 2009;144:308–16.
56. Ley TJ, DeSimone J, Anagnou NP et al. 5-azacytidine selectively increases gamma-globin synthesis in a patient with beta-thalassemia. *N Engl J Med*. 1982;307:1469–75.
57. Veith R, Galanello R, Papayannopoulou T, Stamatoyannopoulos G . Stimulation of F-cell production in patients with sickle-cell anemia treated with cytarabine or hydroxyurea. *N Engl J Med*. 1985;313;1571–75.
58. Hacein-Bey-Abina S, Von Kalle C, Schmidt M et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302:415–19.
59. Hacein-Bey-Abina S, Hauer J, Lim A et al. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*. 2010;363:355–64.
60. Kohn DB. Update on gene therapy for immunodeficiencies. *Clin Immunol*. 2010;135:247–54.
61. Cavazzana-Calvo M, Payen E, Negre O et al. Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature*. 2010;467:318–22.
62. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663–76.
63. Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007;318:1917–20.
64. Lin Y, Chang JC, Lin C, Sun X, Yu J, Kan YW. Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases. *Proc Natl Acad Sci USA*. 2009;106:9826–30.
65. Weatherall DJ. The thalassemias. In: Stamatoyannopoulos G, Nienhaus AW, Leder P, Majerus PW, eds. *The Molecular Basis of Blood Diseases*, 2nd ed. Philadelphia: WB Saunders; 1994:157–206.
66. Malamos B, Fessas P, Stamatoyannopoulos G. Types of thalassemia-trait carriers as revealed by a study of their incidence in Greece. *Br J Haematol*. 1962;8:5–14.
67. Mazza U, Saglio G, Cappio FC et al. Clinical and haematological data in 254 cases of beta-thalassaemia trait in Italy. *Br J Haematol*. 1976;33:91–99.
68. Pootrakul P, Wasi P, Na-Nakorn S. Haematological data in 312 cases of β-thalassemia trait in Thailand. *Br J Haematol*. 1973;24:703–12.
69. Fairbanks VF. *Hemoglobinopathies and Thalassemias: Laboratory Methods and Case Studies*. New York: BC Decker; 1980.
70. Metaxotou-Mavromati AD, Antonopoulou HK, Laskari SS et al. Developmental changes in hemoglobin F levels during the first two years of life in normal and heterozygous beta-thalassemia infants. *Pediatr*. 1982;69:734–38.
71. Weatherall DJ. The thalassemias. In: *Methods in Hematology*. Vol 6. Philadelphia: Churchill Livingstone; 1983.
72. Clegg JB, Weatherall DJ, Milner PF. Haemoglobin Constant Spring: a chain termination mutant? *Nature*. 1971;234:337–40.
73. Weatherall DJ, Clegg JB. α Thalassaemia in association with structural haemoglobin variants and disorders of δ and β chain production. *The Thalassaemia Syndromes*, 3rd ed. Oxford: Blackwell Scientific Publications; 1981: 613–44.
74. Stamatoyannopoulos G, Grosveld F. Hemoglobin switching. In: Stamatoyannopoulos G, Majerus PW, Perlmutter RM, Varmus H, eds. *The Molecular Basis of Blood Diseases*, 3rd ed. Philadelphia: WB Saunders Company; 2001:136–82.
75. Forget BG. Molecular basis of hereditary persistence of fetal hemoglobin. *Ann N Y Acad Sci*. 1998;850:38–44.
76. Thein SL, Wood WG. The molecular basis of β thalassemia, $\delta\beta$ thalassemia and hereditary persistence of fetal hemoglobin. In: Steinberg MH, Forget BG, Higgs DR, Weatherall DJ, eds. *Disorders of Hemoglobin*, 2nd ed. Cambridge: Cambridge University Press, 2009: 323–56.
77. Borg J, Papadopoulos P, Georgitsi M et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. *Nat Genet*. 2010;42:801–5.
78. Zhou D, Liu K, Sun CW et al. KLF1 regulates BCL11A expression and γ- to β-globin gene switching. *Nat Genet*. 2010;42: 742–44.
79. Lettre G, Sankaran VG, Bezerra MAC et al. DNA polymorphisms at the *BCL11A, HBSIL-MYB*, and *β-globin* loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. *Proc Natl Acad Sci USA*. 2008;105:11869–74.
80. Nuinoon M, Makarasara W, Mushiroda T et al. A genome-wide association identified the common genetic variants influence disease severity in β0-thalassemia/hemoglobin E. *Hum Genet*. 2010;127:303–14.
81. Uda M, Galanello R, Sanna S et al. Genome-wide association study shows *BCL11A* associated with persistent fetal hemoglobin and amelioration of the phenotype of β-thalassemia. *Proc Natl Acad Sci USA*. 2008;105:1620–25.
82. Gerald PS, Diamond LK. The diagnosis of thalassemia trait by starch block electrophoresis of the hemoglobin. *Blood*. 1958;13:61–69.
83. Motulsky AG. Frequency of sickling disorders in U.S. blacks. *N Engl J Med*. 1973;288:31–33.
84. Wood WG, Weatherall DJ, Hart GH et al. Hematologic changes and hemoglobin analysis in beta thalassemia heterozygotes during the first year of life. *Pediatr Res*. 1982;16:286–89.
85. Bunn HF, Forget BG. Sickle cell disease: clinical and epidemiological aspects. *Hemoglobin: Molecular, Genetic, and Clinical Aspects*. Philadelphia: WB Saunders;1986:502–64.

86. Embury SH, Clark MR, Monroy G, Mohandas N. Concurrent sickle cell anemia and alpha-thalassemia: effect on pathological properties of sickle erythrocytes. *J Clin Invest*. 1984;73:116–23.
87. de Ceulaer K, Higgs DR, Weatherall DJ et al. Alpha-thalassemia reduces the hemolytic rate in homozygous sickle-cell disease. *N Engl J Med*. 1983;309:189–90.
88. Higgs DR, Aldridge BE, Lamb J et al. The interaction of alpha-thalassemia and homozygous sickle-cell disease. *N Engl J Med*. 1982;306(24):1441–46.
89. Embury SH, Dozy AM, Miller J et al. Concurrent sickle-cell anemia and alpha-thalassemia: effect on severity of anemia. *N Engl J Med*. 1982;306:270–74.
90. Huisman, TH. Combinations of beta-chain abnormal hemoglobins with each other or with beta-thalassemia determinants with known mutations: influence on phenotype. *Clin Chem*. 1997;43:1850–56.
91. Weatherall DJ. Introduction to the problem of hemoglobin E-beta thalassemia. *J Pediatr Hematol Oncol*. 2000;22:551.
92. Winichogoon P, Fucharoen S, Chen P et al. Genetic factors affecting clinical severity of beta-thalassemia syndromes. *J Pediatr Hematol Oncol*. 2000;22:573–80.
93. Fucharoen S, Ketvichit P, Pootrakul P et al. Clinical manifestations of beta-thalassemia/hemoglobin E disease. *J Pediatr Hematol Oncol*. 2000;22:552–57.
94. Johnson CS, Tegos C, Beutler E. Thalassemia minor: routine erythrocyte measurements and differentiation from iron deficiency. *Am J Clin Path*. 1983;80:31–36.
95. Steinberg MH, Dreiling BJ. Microcytosis: its significance and evaluation. *JAMA*. 1983;249:85–87.



15 Megaloblastic and Nonmegaloblastic Macrocytic Anemias

JOEL HUBBARD, PHD
STACEY ROBINSON, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Explain the cause and process of megaloblastic maturation in the bone marrow.
2. Describe the body's requirements for vitamin B_{12} and folate and their physiologic roles.
3. List the laboratory tests used to confirm a diagnosis of cobalamin deficiency and give expected results.
4. List the laboratory tests used to confirm a diagnosis of folic acid deficiency and give expected results.
5. Recognize the six most common disorders associated with a macrocytic anemia.
6. Name four causes of a cobalamin deficiency and give two distinguishing clinical or laboratory characteristics of each.
7. Describe the etiology and pathophysiology of pernicious anemia, including clinical symptoms and clinical subtypes.
8. Name three causes of a folate deficiency and give two distinguishing clinical or laboratory characteristics of each.
9. Differentiate the pathophysiology and peripheral blood findings of non-megaloblastic macrocytic anemia from those of megaloblastic anemias.
10. Summarize the typical blood picture seen with a folate or cobalamin deficiency.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Summarize the process of cobalamin and folic acid metabolism and explain how a deficiency can result in megaloblastosis.
2. Compare macrocytosis associated with a normoblastic marrow and macrocytosis associated with a megaloblastic marrow on the basis of physiological defect, and differentiate them based on the laboratory blood picture.
3. Summarize the mechanism of maturation defects that lead to megaloblastosis and recognize the morphologic blood cell abnormalities.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

4. Compare and contrast the various clinical forms and causes of a cobalamin and folate deficiency on the basis of clinical symptoms and laboratory results.
5. Categorize the causes and clinical variations of pernicious anemia.
6. Compare and contrast the various clinical forms and causes of a folic acid deficiency.
7. Choose and briefly explain four laboratory tests used to identify the cause of a macrocytic anemia; give the expected results of these four tests in a patient with an autoantibody directed against intrinsic factor.
8. Assess Schilling's test results and provide a differential diagnosis.
9. Compare and contrast the causes of macrocytosis that have a normoblastic marrow.
10. Construct an algorithm of laboratory testing to distinguish between a megaloblastic anemia and a macrocytic, normoblastic anemia.
11. Evaluate a case study from a patient with anemia. Determine the most probable diagnosis from the medical history and laboratory results.

Key Terms

Achlorhydria
Cobalamin
Demyelination
Dyspepsia
Folate
Folic acid
Glossitis
Intrinsic factor (IF)
Megaloblastic
Nuclear-cytoplasmic asynchrony
Pernicious anemia (PA)
Schilling test

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the maturation process of erythrocytes in the marrow. (Chapter 5)
- Outline the functional and morphologic classification of anemia and list the basic laboratory tests to diagnose anemia. (Chapter 11)

Level II

- Summarize the concepts of cell development, regulation, and the process of cell division. (Chapter 2)
- List and describe the laboratory tests used in differential diagnosis of anemia. (Chapter 11)

CASE STUDY

We will refer to this case study throughout the chapter.

Kathy, a 36-year-old female, experienced a recent 35-lb weight loss. Her tongue was red and fissured. She also complained of chronic fatigue and shortness of breath upon exertion. Physical examination suggested signs of jaundice and increased numbness and a tingling sensation of fingers and toes. She was hospitalized with the general diagnosis of moderate anemia, jaundice, and neurological symptoms. Her admitting CBC demonstrated the following laboratory results:

		Differential	
WBC	4.5×10^9/L	Lymphs	36.0%
RBC	2.50×10^{12}/L	Monos	3.6%
Hb	10.0 g/dL (100.0 g/L)	Neutrophils	59.4%
Hct	31% (0.31 L/L)	Eosinophils	1.0%
MCV	124 fL	Basophils	0.0%
MCH	40.50 pg	NRBCs/100 WBCs	5.0%
MCHC	32.70 g/dL	Moderate hypersegmented neutrophils	
RDW	21.20		
PLT	155×10^9/L		

The following abnormal erythrocyte morphology was reported:

Macrocytes	2+
Anisocytosis	3+
Poikilocytosis	2+
Ovalocytes	1+
Basophilic stippling	1+
Occasional Howell-Jolly bodies	

Consider the reflex tests that might be important in identifying the etiology of this anemia.

OVERVIEW

This chapter is a study of the macrocytic anemias, which can be megaloblastic or nonmegaloblastic. The first part of the chapter discusses the megaloblastic anemias beginning with a description of the clinical and laboratory findings. Because megaloblastic anemia is most often due to deficiencies or abnormal metabolism of folate or cobalamin (vitamin B_{12}), the metabolism of these vitamins is discussed in detail. The latter part of the chapter reviews the causes of nonmegaloblastic macrocytic anemia and compares the laboratory test results in nonmegaloblastic and megaloblastic anemia. The laboratory professional can often identify diagnostic clues of megaloblastic anemia on review of a blood smear.

INTRODUCTION

Macrocytic anemias are characterized by large erythrocytes (mean MCV >100 fL) with an increased MCH and a normal hemoglobin content (MCHC). This is an important group of anemias because macrocytosis is frequently a sign of a disease process that can result in significant morbidity if left untreated.

Macrocytosis is found in 2.5–4.0% of adults who have a routine complete blood count.[1] In up to 60% of cases, macrocytosis is not accompanied by anemia,[2] but isolated macrocytosis should always be investigated. Macrocytosis without anemia can indicate early folate or cobalamin (vitamin B_{12}) deficiency because macrocytosis precedes the development of anemia in these disorders.

Macrocytosis detected by automated cell counters is not always apparent microscopically on stained blood smears. In some cases, the erythrocyte size on automated counters is falsely elevated due to hyperglycemia, cold agglutinins, and extreme leukocytosis. These causes of false macrocytosis need to be differentiated from true macrocytosis.

The most common cause of true macrocytosis is alcoholism. Other causes include folate and cobalamin deficiencies, drugs including chemotherapy, reticulocytosis due to hemolysis or bleeding, myelodysplasia, liver disease, and hypothyroidism.[2]

Macrocytic anemias are generally classified as megaloblastic or nonmegaloblastic (normoblastic), depending on morphologic characteristics of erythroid precursors in the bone marrow (Table 15-1 ★). The **megaloblastic** anemias are the result of a defect in DNA synthesis. Frequently there is an arrest in the S phase of the cell cycle and to a lesser extent during other phases of the cell cycle due to delayed nuclear development. RNA and protein synthesis, however, are relatively unimpaired. The result is unbalanced cell growth and impaired cell division characterized by erythroblasts with distinct abnormal morphologic features. The nucleus appears immature with a fine particulate chromatin pattern, whereas the cytoplasm is increased and relatively more mature (referred to as **nuclear-cytoplasmic asynchrony**). These cells are referred to morphologically as *megaloblastic*. All proliferating cells are affected.

The basis for the nonmegaloblastic anemias is not always as well defined but is often related to an increase in membrane lipids. The macrocytes in nonmegaloblastic macrocytic anemia are usually round, but in megaloblastic anemia, they are oval (Figure 15-1a, b ■). A flow chart for laboratory analysis to help distinguish causes of macrocytic anemia is shown in Figure 15-2 ■.

★ **TABLE 15-1** Conditions Associated with Megaloblastic and Nonmegaloblastic (Normoblastic) Macrocytic Anemias

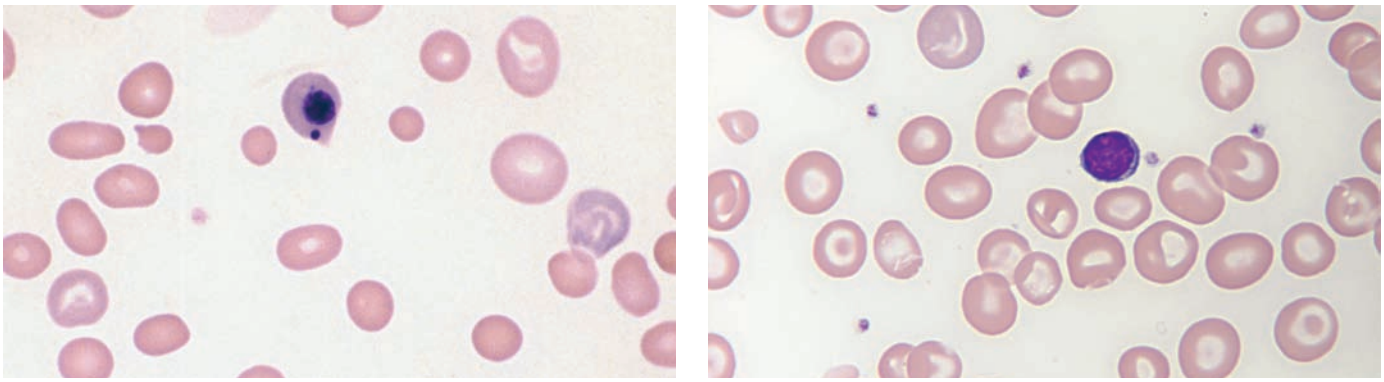
Megaloblastic	Normoblastic
Folate deficiency	Alcoholism
Nutritional deficiency	Liver disease
Increased requirement (pregnancy)	Shift reticulocytosis in hemolytic anemia or hemorrhage
Intestinal malabsorption	Hypothyroidism
Drug inhibition	Aplastic anemia
Cobalamin deficiency	Obstructive jaundice
Pernicious anemia	Splenectomy
Small bowel resection	Pregnancy
Gastrectomy	Artifactual:
Intestinal malabsorption	hyperglycemia
Nutritional deficiency	cold agglutinins
Increased requirement (pregnancy)	leukocytosis
Transcobalamin deficiency	
Nitrous oxide abuse	
Other causes	
Chemotherapy with metabolic inhibitors	
Orotic aciduria	
Congenital dyserythropoietic anemia (CDA)	

MEGALOBLASTIC ANEMIA

Although very little was known about the function or origin of blood cells before the twentieth century, some perceptive individuals began to make associations between anemia and other clinical signs in patients. In 1822, J. S. Coombe, a Scottish physician, made the initial clinical description of a patient who appeared to have megaloblastic anemia. He was the first to suggest that this anemia might be related to **dyspepsia**.[3] In 1855, Thomas Addison reported his description of a macrocytic anemia, but he made no reference to the typical microscopic blood findings.[4] The discovery and description of the abnormal erythroid precursors in the bone marrow associated with this anemia were made possible by the advent of triacid stains. Paul Ehrlich is credited with coining the term *megaloblast* in 1891 to describe the large abnormal precursors in megaloblastic anemia.[5]

Megaloblastic anemia is classified as a nuclear maturation defect. Anemia is attributed primarily to a large degree of ineffective erythropoiesis resulting from disrupted DNA synthesis. The anemia was called *megaloblastic* in an attempt to describe the giant, abnormal-appearing erythroid precursors (megaloblasts) in the bone marrow. The generic word *megaloblast* describes any maturation stage of the megaloblastic erythroid series (i.e., polychromatic megaloblast). Other nucleated cells of the marrow are also typically abnormal.

About 95% of megaloblastic anemias are caused by deficiencies of either vitamin B_{12} (**cobalamin**) or **folic acid**, vitamins necessary as coenzymes for nucleic acid synthesis. In the majority of cases, cobalamin deficiency is secondary to a deficiency of **intrinsic factor (IF)**, a protein necessary for absorption of cobalamin, rather than to a nutritional



a b

FIGURE 15-1 (a) Peripheral blood film from a patient with pernicious anemia. Note the anisocytosis with oval macrocytes and the nucleated red blood cell with a Howell-Jolly body. (Wright-Giemsa stain; 1000× magnification). (b) Peripheral blood film from a patient with normoblastic, macrocytic anemia. Compare the size of the RBCs with the lymphocyte. The RBCs are primarily round macrocytes. (Wright-Giemsa stain, 1000× magnification).

deficiency of the vitamin. Folic acid deficiency, on the other hand, is most often due to an inadequate dietary intake. Inherited disorders affecting DNA synthesis or vitamin metabolism are rare causes of megaloblastosis.

☑ CHECKPOINT 15-1

Explain why patients with cobalamin or folate deficiency have megaloblastic maturation.

Clinical Findings

The onset of megaloblastic anemia is usually insidious; because the anemia develops slowly, it produces few symptoms until the hemoglobin and hematocrit are significantly depressed. Patients can present with typical anemic symptoms of lethargy, weakness, and a yellow or waxy pallor. Dyspeptic symptoms are common. **Glossitis** with a beefy red tongue, or more commonly a smooth pale tongue, is characteristic. Loss of weight and loss of appetite are common complaints. In pernicious anemia (see "Pernicious Anemia"), atrophy of the gastric parietal cells causes decreased secretion of intrinsic factor and hydrochloric acid. Bouts of diarrhea can result from epithelial changes in the gastrointestinal tract.

Neurological disturbances occur only in cobalamin deficiency, not in folic acid deficiency. They are the most serious and dangerous clinical signs because neurological damage can be permanent if the deficiency is not treated promptly. The patient's initial complaints occasionally are related to neurological dysfunction rather than to anemia. Neurological damage has been reported to occur even before anemia or macrocytosis in some cases, particularly in elderly people. The bone marrow, however,

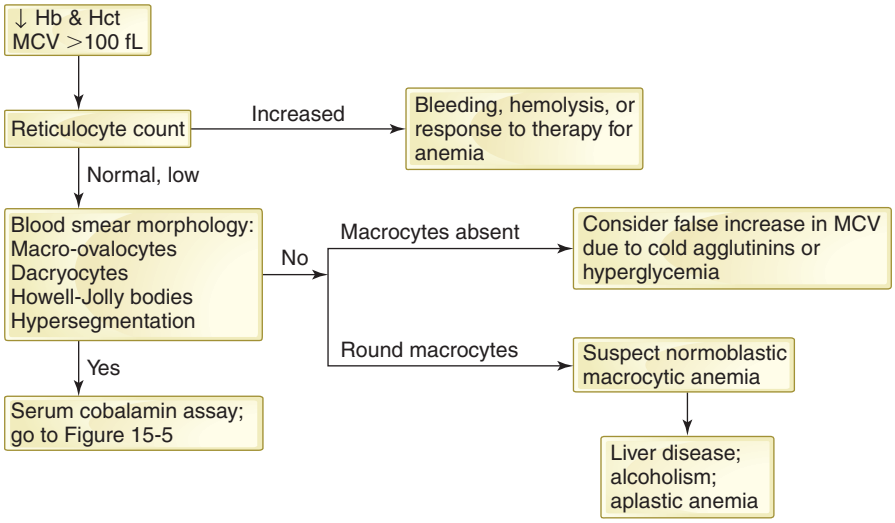


FIGURE 15-2 Algorithm for the differential diagnosis of the megaloblastic anemias from other macrocytic anemias.

Hb = hemoglobin; Hct = hematocrit; MCV = mean cell volume

usually reveals megaloblastic changes even in the absence of anemia. Tingling, numbness, and weakness of the extremities reflect peripheral neuropathy. Loss of vibratory and position (proprioceptive) sensations in the lower extremities can cause the patient to have an abnormal gait. The patient's relatives sometimes note mental disturbances such as loss of memory, depression, and irritability. *Megaloblastic madness* is a term used to describe severe psychotic manifestations of cobalamin deficiency. A patient with severe anemia occasionally is asymptomatic, which is probably a reflection of a very slowly developing anemia. It has been suggested that cobalamin deficiency should be suspected in all patients who have an unexplained anemia and/or neurological disturbances or in individuals who are at risk of developing a deficiency such as elderly people or those with intestinal diseases.[6]

☑ CHECKPOINT 15-2

Patients with megaloblastic anemia often present with a yellow or waxy pallor. What is the diagnostic significance of this clinical symptom?

Laboratory Findings

Laboratory tests are critical to a diagnosis of megaloblastic anemia. The routine CBC with a review of the blood smear gives important diagnostic clues and helps in selecting reflex tests.

Peripheral Blood

Megaloblastic anemia is typically a macrocytic, normochromic anemia. The MCV is usually >100 fL and can reach a volume of 140 fL. However, an increased MCV is not specific for megaloblastic anemia. The MCH is increased because of the large cell volume, but the MCHC is normal. In cobalamin deficiency, a macrocytosis can precede the development of anemia by months to years.[7–9] On the other hand, the MCV can remain within the reference interval. Epithelial changes in the gastrointestinal tract can cause iron absorption to be impaired. If an iron deficiency (which characteristically produces a microcytic, hypochromic anemia) coexists with megaloblastic anemia, macrocytosis can be masked, and the MCV can be in the normal range.[10] Other conditions that have been shown to coexist with megaloblastic anemia in the absence of an increased MCV include thalassemia, chronic renal insufficiency, and chronic inflammation or infection.[9] Sometimes these coexisting causes of anemia are not recognized until after the megaloblastic anemia has been treated. It has been suggested that if coexisting iron deficiency, thalassemia, or chronic disease is suspected, patient medical history, racial/ethnic background, and previous MCV should be considered.[11]

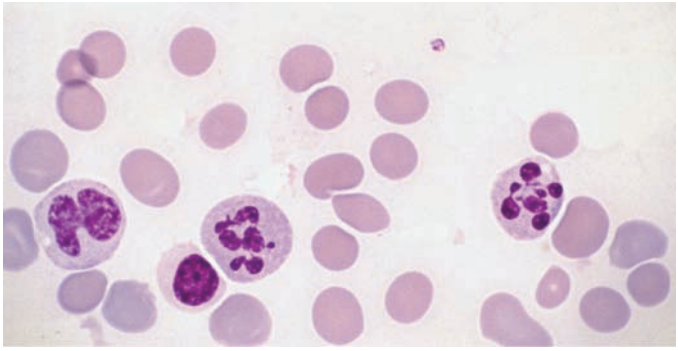
Hematologic parameters vary considerably (Table 15-2 ★). The hemoglobin and erythrocyte count range from normal to very low. The erythrocyte count is occasionally $<1 \times 10^{12}$/L. However, anemia is not always evident. In one study of 100 patients with confirmed cobalamin deficiency, only 29% had a hemoglobin of <12 g/dL.[12] This is significant because neurologic symptoms can be present even if the MCV and/or hematocrit are normal.[13] Because the abnormality is a nuclear maturation defect, the megaloblastic anemias affect all three blood cell lineages: erythrocytes, leukocytes, and platelets. This is unlike most other anemias that typically involve only erythrocytes. The leukocyte count can be decreased due to an absolute neutropenia. Platelets can also be decreased but do not usually fall below 100×10^9/L. The relative reticulocyte count (percentage) is usually normal; however, because of the severe anemia, the corrected reticulocyte count is <2%, the absolute reticulocyte count is low, and RPI is <2 (Chapter 11).

The distinguishing features of megaloblastic anemia on the stained blood smear include the triad of oval macrocytes (macro-ovalocytes), Howell-Jolly bodies, and hypersegmented neutrophils (Figure 15-1a). Anisocytosis is moderate to marked with normocytes

★ **TABLE 15-2** Comparison of Common Laboratory Values in Megaloblastic and Nonmegaloblastic Macrocytosis

Laboratory Value	Megaloblastic Macrocytosis	Nonmegaloblastic Macrocytosis
WBC count	Decreased	Normal
Platelet count	Decreased	Normal
RBC count	Decreased	Decreased
Hemoglobin	Decreased	Decreased
Hematocrit	Decreased	Decreased
MCV	Usually >110 fL	>100 fL
RBC morphology	Ovalocytes, Howell-Jolly bodies, polychromasia	Polychromasia, target cells, and stomatocytes (liver disease), schistocytes (hemolytic anemias)
Hypersegmentation of neutrophils	Present	Absent
Reticulocyte count	Normal to decreased	Normal, decreased, or increased
Serum cobalamin	Decreased in cobalamin deficiency	Usually normal
Serum folate	Decreased in folate deficiency	Normal (except in alcoholism when it can be decreased)
FIGLU	Increased in folate deficiency	Normal
MMA	Increased in B_{12} deficiency	Normal
Homocysteine	Increased	Normal
Serum bilirubin	Increased	Normal to increased
LD	Increased	Normal to increased

MCV = mean corpuscular volume; FIGLU = formiminoglutamic acid; MMA = methylmalonicacid; LD = lactic dehydrogenase



■ **FIGURE 15-3** Hypersegmented neutrophils from the peripheral blood of a patient with pernicious anemia. (Wright-Giemsa stain; 1000× magnification)

and a few microcytes in addition to the macrocytes. Poikilocytosis can be striking and is usually more so when the anemia is severe. Polychromatophilia and megaloblastic erythroblasts can be seen, especially when the anemia is severe, indicating the futile attempt of the bone marrow to increase peripheral erythrocyte mass. Cabot rings occasionally can be seen in erythrocytes.

Granulocytes and platelets can also show changes evident of abnormal hematopoiesis. Hypersegmented neutrophils can be found in megaloblastic anemia even in the absence of macrocytosis (Figure 15-3 ■). Finding 5% or more neutrophils with five lobes or one neutrophil with six or more lobes is considered hypersegmentation. This finding of hypersegmented neutrophils is considered highly sensitive and specific for megaloblastic anemia. Therefore, hypersegmented neutrophils offer an important clue to megaloblastic anemia in the face of a coexisting disease that tends to keep erythrocyte volume <100 fL. One study showed that in patients with renal disease, iron deficiency, or chronic disease with a normal or decreased MCV and 1% hypersegmented neutrophils, 94% had vitamin B_{12} or folic acid deficiency.[8] If 5% hypersegmented neutrophils were counted, the incidence of the vitamin B_{12} or folic acid deficiency increased to 98%. Hypersegmented neutrophils tend to be larger than normal neutrophils. A mild shift to the left with large hypogranular bands can also be noted. Platelets can be large, especially when the platelet count is decreased.

☑ CHECKPOINT 15-3

Why are abnormalities of leukocytes and platelets present in megaloblastic anemia?

Bone Marrow

If physical examination, patient history, and peripheral blood findings suggest megaloblastic anemia, a bone marrow examination can help establish a definitive diagnosis. In megaloblastic states, the bone marrow is hypercellular with megaloblastic erythroid precursors and a decreased M:E ratio. In a long-standing anemia, red marrow can expand into the long bones. About half the erythroid precursors typically show megaloblastic changes. Megaloblasts are large nucleated erythroid precursors that display nuclear-cytoplasmic asynchrony with nuclear maturation lagging behind cytoplasmic maturation (Figure 15-4 ■). The nucleus of the megaloblast contains loose, open chromatin that stains poorly; cytoplasmic development continues in a normal fashion. At each stage of development, the cells contain more cytoplasm with a more mature appearance relative to the size and maturity of the nucleus (resulting in a decreased nuclear:cytoplasmic [N:C] ratio).

The megaloblastic features are more easily noted in later stages of erythroid development, especially at the polychromatophilic stage in which the presence of hemoglobin mixed with RNA gives the cytoplasm the gray-blue color typical of this erythroid precursor. The polychromatophilic megaloblast nucleus, however, still has an open (lacy) chromatin pattern more typical of an earlier stage of development.

Leukocytes and platelets also show typical features of a nuclear maturation defect as well as ineffective leukopoiesis and thrombopoiesis. Giant metamyelocytes and bands with loose, open chromatin in the nuclei are diagnostic (Figure 15-4a). The myelocytes can show poor granulation as do more mature stages. Megakaryocytes can be decreased, normal, or increased. Maturation, however, is distinctly abnormal. Larger than normal forms can be found with separation of nuclear lobes and nuclear fragments.

CASE STUDY (continued from page 278)

Based on the initial CBC results, further testing was ordered with the following results:

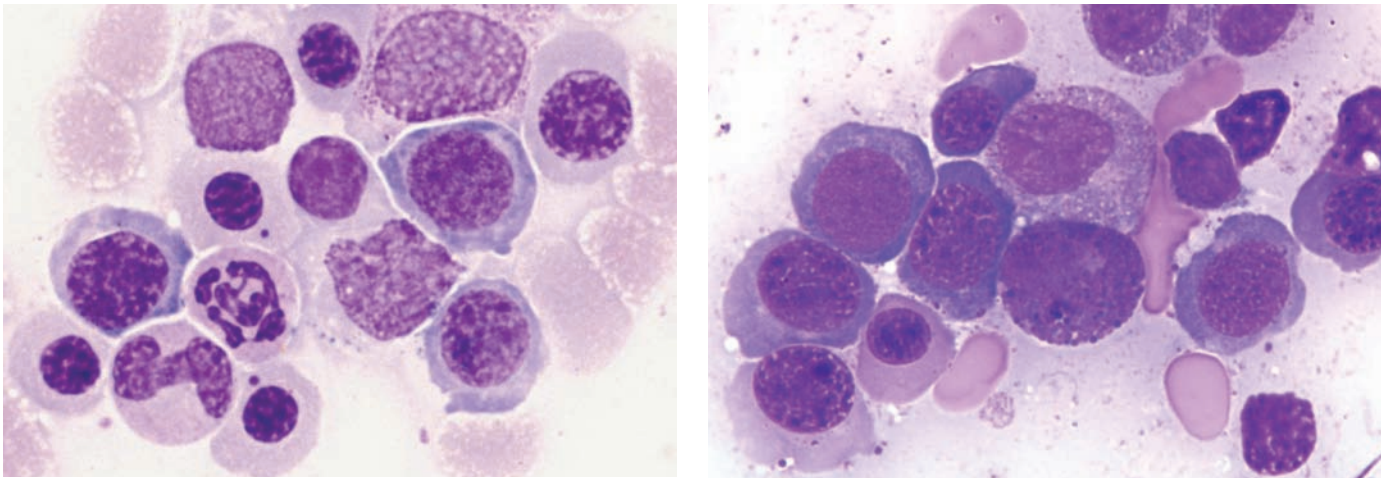
B_{12} (cobalamin)	50 pg/mL	Low
Folate	10.3 ng/mL	Normal
Total billirubin	2.5 mg/dL	High
Direct billirubin	0.8 mg/dL	Normal
AST	35 U/mL	Normal
ALT	30 U/mL	Normal

Examination of a bone marrow aspirate revealed an erythroblastic hyperplasia with megaloblastic erythroblasts.

1. What is the morphologic classification of the patient's anemia?
2. Based on the information obtained so far, what is the most likely defect?
3. What is the significance of the AST/ALT results?
4. What further testing can be done to obtain a definitive diagnosis?

Other Laboratory Findings

If CBC results suggest megaloblastic anemia, further testing is necessary to distinguish the cause. Although no major medical organization has published guidelines for reflex testing, the most common next step is to measure serum cobalamin and serum or red cell folate. Laboratories use different methods (chemiluminescence, radioassay) to measure cobalamin, so there is no "gold standard" to use as a reference interval. Generally values <150 pg/mL are consistent with cobalamin deficiency, whereas levels >400 pg/mL suggest adequate cobalamin. Borderline levels (150–400 pg/mL) can be associated with cobalamin deficiency.



a b

■ **FIGURE 15-4** **(a)** Basophilic and orthochromatic megaloblasts in the bone marrow from a patient with pernicious anemia. Note the large size of the cells, the open chromatin network in the nuclei, and the presence of Howell-Jolly bodies in the orthochromatic megaloblasts. Note the nuclear-cytoplasmic asynchrony. There is a large band neutrophil and a segmented neutrophil with at least five nuclear lobes. (Bone marrow; Wright-Giemsa stain; 1000× magnification). **(b)** Nuclear-cytoplasmic asynchrony in the bone marrow. Note the large size of the cells (bone marrow; Wright-Giemsa stain; 1000× magnification).

Measurement of erythrocyte folate is not influenced as much by recent dietary changes as is serum folate and gives an accurate estimate of the average folate levels over the preceding several months.[14,15] On the other hand, if there is a cobalamin deficiency, folate will leak out of the cells, which will give a false low red cell folate and false increased serum folate. In addition, red cell folate is measured by folate-binding protein assays that rely on chemiluminescence methodology. These methods show considerable analytic variability. Therefore, the less expensive serum folate measurement is preferred for initial testing. If serum folate is >4 ng/mL, folate deficiency can be ruled out.

Early megaloblastic changes can be detected by testing for methylmalonic acid (MMA) and homocysteine levels in the blood. Tests for these metabolites are intermediates in folate and cobalamin metabolism and are elevated early in functional vitamin deficiencies. Tests for these metabolites are more sensitive than serum cobalamin levels and increase earlier than a drop in the cobalamin level. By performing tests for both MMA and homocysteine, it is possible to differentiate cobalamin deficiency from folate deficiency. Homocysteine is elevated in folate deficiency, whereas MMA is usually normal. On the other hand, both homocysteine and MMA are elevated in cobalamin deficiency. An increase in both MMA and homocysteine is also found in combined cobalamin and folate deficiencies. In these cases, clinical information is important to help establish a differential diagnosis. A block in the metabolism of histidine to glutamic acid occurs in folic acid deficiency and causes increased urinary excretion of formiminoglutamic acid (FIGLU), an intermediate metabolite, after the administration of histidine. These metabolites return to normal levels when the appropriate vitamin is given to the patient. It is recommended that clinicians first use the lower cost tests of serum cobalamin and serum folate to diagnose cobalamin and folate deficiencies and use the higher cost MMA and homocysteine tests if cobalamin and folate test results are not definitive[16] (Figure 15-5 ■).

The large degree of ineffective erythropoiesis results in hemolysis in the marrow and an increase in plasma iron turnover, serum iron, indirect bilirubin, and urobilinogen. The characteristic marked increase in fractions 1 and 2 of serum lactic dehydrogenase (LD) is partially caused by the destruction of megaloblasts rich in LD. The increase is roughly proportional to the degree of anemia. Haptoglobin, uric acid, and alkaline phosphatase are decreased. Additional tests are discussed in the following sections.

☑ CHECKPOINT 15-4

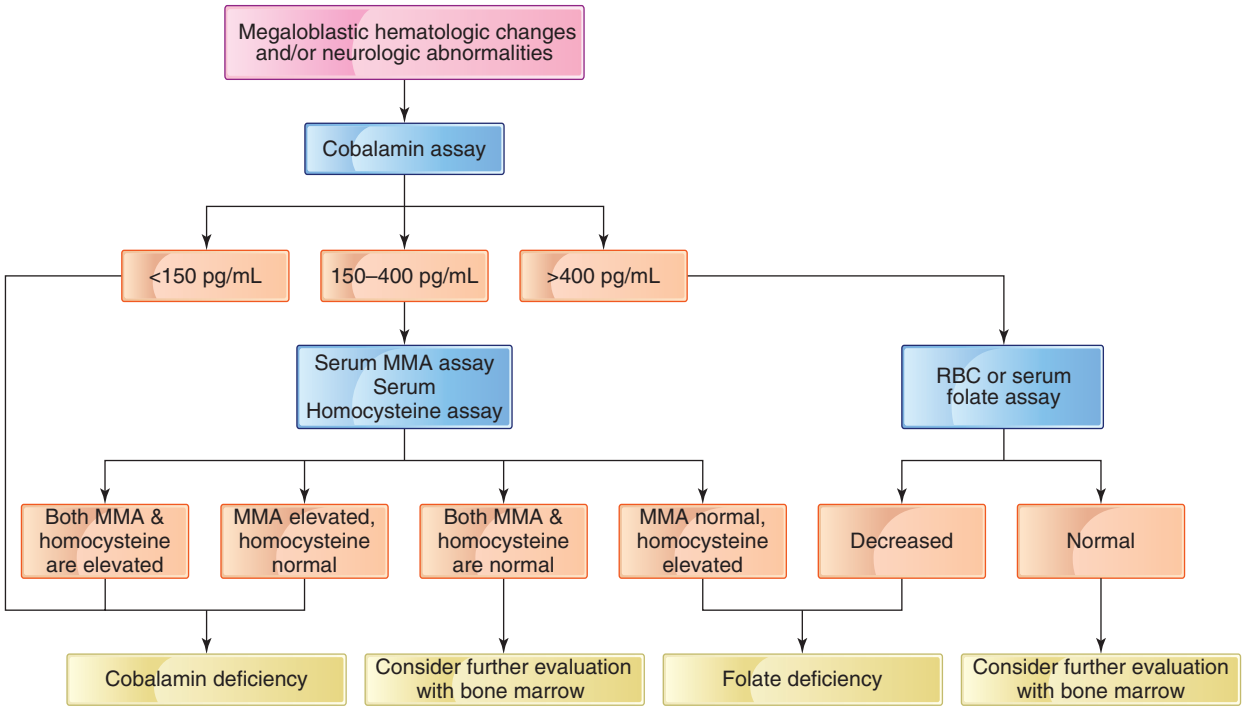
What abnormal morphological findings on a stained blood smear compose the triad in megaloblastic anemia?

Folate

A folate deficiency must be considered in the differential diagnosis of macrocytosis associated with megaloblastic anemia.

Structure and Function

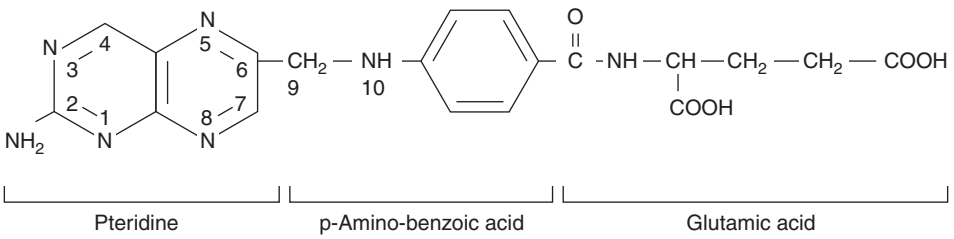
Folic acid is the parent substance of a large group of compounds known as *folates*. Chemically, folic acid is known as *pteroylmonoglutamate (PteGlu)*. Structurally, folic acid is composed of three parts: (1) pteridine, a nitrogen-containing ring, (2) a ring of p-amino-benzoic acid, and (3) a glutamic acid residue (Figure 15-6 ■). The term *folic acid* refers to this base structure (inert form) and to the commercially available synthetic form used for the fortification of food supplies and for dietary supplements. Folic acid itself does not occur naturally. Folates can vary in their oxidation status, their one-carbon substituent unit, and in their number of glutamic acid residues. Tetrahydrofolate (THF or FH_4), the active form, is produced by a four-hydrogen reduction of the pteridine ring. Dihydrofolate (DHF) is an intermediate in this reaction.



■ **FIGURE 15-5** Algorithm that can be used to determine the cause of megaloblastic anemia using laboratory tests. Analysis can begin with serum cobalamin assay. If results are in the low normal range (150–400 pg/mL) or the patient has unexplained neurologic symptoms, measurement of cobalamin metabolic intermediates, methylmalonic acid (MMA), and homocysteine is suggested. If both are elevated, cobalamin deficiency is considered. If MMA is normal and homocysteine is elevated, folate deficiency is probable. RBC or serum folate also can be measured, especially if cobalamin is within the reference interval. If these test results conflict with the clinical diagnosis, therapeutic trials with cobalamin can be used. Bone marrow is rarely performed for diagnostic purposes but can be necessary in some cases. The bone marrow shows megaloblastic features in both folate and cobalamin deficiencies.

The function of THF is to transfer one-carbon compounds from donor molecules to acceptor molecules in intermediary metabolism. In this capacity, folate serves a vital role in the metabolism of nucleotides and amino acids:

1. The carbon transfer reaction involved in the de novo synthesis of DNA is initiated when the carbon side chain of serine is transferred to THF to form N^5, N^{10}-methylene THF (Figure 15-7a ■; Table 15-3 ★). This carbon is then transferred to deoxyuridilate (dUMP) to form deoxythymidylate (dTMP), a pyrimidine of DNA. In this reaction, THF is oxidized to DHF. The DHF is reduced back to THF by DHF reductase and then to N^5, N^{10}-methylene THF by the serine reaction discussed earlier. A deficiency of folate or cobalamin blocks the synthesis of the nucleotide dTTP. Folates also transport formate (as N^{10}-formyl THF) for purine synthesis.
2. The metabolism of histidine to glutamic acid requires THF (Figure 15-7b ■, Table 15-3). The intermediate metabolite of this reaction is formiminoglutamic acid (FIGLU), which requires THF for conversion to glutamic acid. A deficiency of folate blocks this reaction, resulting in an increase in FIGLU excretion.
3. The synthesis of methionine from homocysteine requires the donation of a methyl group from N^5-methyl THF and the action of cobalamin as a cofactor, resulting in methionine and THF (Figure 15-7a). A deficiency of either folate or cobalamin blocks this reaction, resulting in an increase in homocysteine levels.



■ **FIGURE 15-6** Molecular structure of the folic acid molecule.

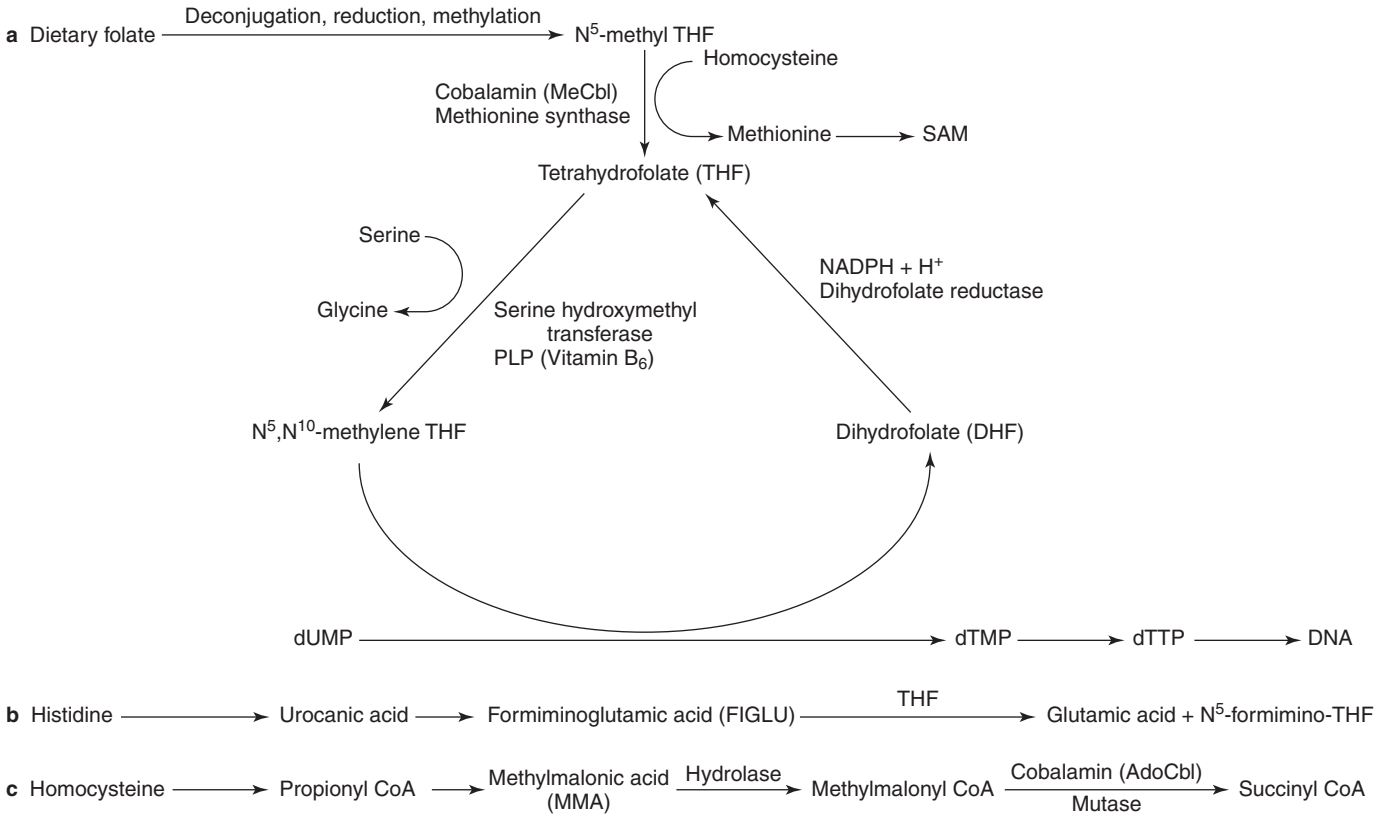
★ **TABLE 15-3** Metabolic Reactions Requiring Folic Acid Coenzymes

System	Reaction
Serine ↔ Glycine	Ser + THF ↔ N^5, N^{10}-methylene THF + Gly
Thymidylate synthesis	dUMP + N^5,N^{10}-methylene THF → DHF + dTMP
Histidine catabolism	Formininoglutamate + THF → N^5-formimino THF + glutamic acid
Methionine synthesis	Homocysteine + N^5-methyl THF → THF + Methionine

Metabolism

Folate is present in food and is synthesized by microorganisms. Most folate in food is in the conjugated polyglutamate form. It is deconjugated in the intestine to a monoglutamate prior to absorption. Absorption can take place throughout the small intestine but is especially significant in the proximal jejunum. Once taken up by the intestinal epithelial cell, the folate is reduced to N^5-methyl THF, the primary circulating form of THF in the blood. N^5-methyl THF is distributed throughout the body via the blood and attaches to cells by means of specific receptors called *cell surface folate receptor-α* (a glycosyl-phosphatidylinositol [GPI]-anchored receptor; Chapter 17). This receptor is upregulated through transcriptional, translational, and post-translational mechanisms when intracellular and extracellular folate is decreased. Translational receptor upregulation involves the covalent binding of accumulated homocysteine (which occurs when there is a folate deficiency) with a protein called *heterogeneous nuclear ribonucleoprotein-E1* (hnRNP-E1).[17]

Once inside the cell, N^5-methyl THF must be demethylated and reconjugated by the addition of seven or eight glutamic acid residues to keep it from leaking out again. Demethylation is a reaction that



■ **FIGURE 15-7** Biochemical reactions using folic acid, cobalamin, and derivatives. **(a)** The role of folate and cobalamin in the synthesis of DNA. A deficiency of methylcobalamin causes a failure of methylation of homocysteine, which leads to a reduction in tetrahydrofolate (THF) and the trapping of the folate as N^5-methyl THF. This causes a deficiency of N^5,N^{10}-methylene THF, a coenzyme needed in the synthesis of dTTP, a nucleotide of DNA. (PLP = pyridoxal-5-phosphate) **(b)** The role of folate in the catabolism of histidine. **(c)** The role of vitamin B_{12} (adenosyl-cobalamin) in the conversion of methylmalonic acid (MMA) to succinyl CoA. With deficiencies of cobalamin and/or folate, the intermediates MMA, homocysteine, and FIGLU are trapped and increase in concentration. Tests to detect cobalamin deficiency include direct measurement of cobalamin and tests to detect increased MMA and homocysteine, metabolic intermediates that require cobalamin for metabolism. Tests to detect folic acid deficiency include direct measurement of folate or measurement of homocysteine and FIGLU, intermediates that require folate for metabolism.

requires cobalamin and methionine synthase (Figure 15-7a). Thus, a deficiency of cobalamin traps folate in its methylated form and blocks the formation of other forms of THF. This is commonly referred to as the *folate trap* or *methyl trap*. Although free THF is easily conjugated within cells, methyl-THF is not; consequently, much of the methyl-THF taken up by a cobalamin-deficient cell leaks out before additional glutamates can be added.[14] The cells in cobalamin deficiency are unable to retain their folate, leading to tissue folate depletion. In the demethylation of N^5-methyl THF by cobalamin, homocysteine is methylated to methionine, a precursor of S-adenosylmethionine (SAM) (Table 15-3). This reaction requires methionine synthase and cobalamin. SAM is thought to be critical to nervous system function.

Requirements

Folate is present in most foods including eggs, milk, yeast, mushrooms, and liver but is especially abundant in green leafy vegetables (from which it gets its name). It is also synthesized by microorganisms. The vitamin is destroyed by heat; thus, when food is overcooked, much of the folate is destroyed. Ascorbate protects folate from oxidation and, when present, can protect folate to some extent from heat degradation. The recommended daily dietary allowance of food folic acid for adults is ~400 mcg (μg), of which about 50–80% is absorbed in the intestine. This is adequate to provide the minimum daily requirement of ~50 mcg/day needed to sustain normal metabolism. The liver stores from 5 to 10 mg of folate, which is sufficient to provide the daily requirement for three to six months if folate is omitted from the diet.

Folate plays an important role in normal embryogenesis. The folate receptor-α is activated early in embryonic stem cells and increases as the need for folate increases. Observations have revealed a high incidence of low folate levels in women who give birth to babies with neural tube defects (NTD) compared with women who give birth to normal babies. Experimental studies suggest perturbations in folate receptor-α may be involved in these neural tube closure abnormalities.[17] Several studies have shown that supplementation of folate during pregnancy can reduce the rate of occurrence of these birth defects by as much as one-half. In an effort to reduce NTDs, the U.S. government required the fortifications of grain products with folic acid beginning in the fall of 1997.[18] The goal was to increase the daily dietary folate to 100 mcg per person. It has been suggested that pregnant women need an intake of about 800 mcg/day.

Folate can become depleted quickly in conditions with rapid cell turnover such as sickle cell anemia and other hemolytic anemias, during growth, pregnancy, and lactation. Thus, the daily requirement in patients with these conditions is increased.

☑ CHECKPOINT 15-5

Hal had small bowel resection due to carcinoma. Explain why he is at high risk for folate deficiency.

Pathophysiology of Folate Deficiency

Folate deficiency results in decreased synthesis of N^5, N^{10}-methylene THF, which is needed as a cofactor in DNA synthesis. Consequently, there is a marked slowing of DNA synthesis, and the S phase of the cell cycle is prolonged. The impairment of DNA synthesis is due to the inability to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), the precursor of dTTP. Subsequently, the dUMP is phosphorylated to the triphosphate form (dUTP). DNA polymerase does not effectively distinguish dUTP from dTTP, and dUTP is erroneously incorporated into the DNA of folate-deficient cells. The DNA "proof-reading" function of the cells recognizes the mistake and tries to repair the DNA by replacing uridine with thymidine, but the repair attempt fails due to the lack of available dTTP. The result is ultimately DNA fragmentation and cell death by apoptosis.[14] All rapidly dividing cells—including erythrocyte, leukocyte, and platelet precursors and intestinal epithelium—are affected by a folate deficiency. Surviving hematopoietic cells show characteristic megaloblastic changes.

The bone marrow can exhibit a three-fold increase in erythropoiesis, but the peripheral blood reticulocyte count is low, indicating a large degree of ineffective erythropoiesis. Increased apoptosis of the blood cell precursors leads to increased heme catabolism and iron turnover, signs of hemolysis, jaundice, and pancytopenia. Extramedullary hemolysis also occurs, and circulating red cell survival can be decreased by 30–50%. The compounds requiring folic acid as a cofactor for metabolism (homocysteine and FIGLU) accumulate. Mildly elevated levels of homocysteine are considered a major risk factor for atherosclerosis and venous thrombosis (Chapter 35).

The clinical findings of folate deficiency develop sequentially. Serum folate decreases within 1 to 2 weeks of onset of a folate deficiency. Hypersegmented neutrophils are the first morphologic change and occur at about 2 weeks. The urinary excretion of FIGLU increases next at about 13 weeks, and anemia appears last at about 19–20 weeks.

Causes of Folate Deficiency

Folate deficiency can occur as the result of an inadequate dietary intake, an increased requirement, malabsorption in the small intestine, or drug inhibition (Table 15-4 ★).

Inadequate Diet

The most common cause of folate deficiency is an inadequate dietary intake of folic acid. This is seen most often in poor and elderly people who fail to obtain enough of the appropriate foods to maintain adequate folic acid intake. People who are alcoholics whose diet consists mainly of large quantities of ethanol have a deficiency of many vitamins in addition to folic acid. Complicating the folate deficiency in

★ **TABLE 15-4** Causes of Folate Deficiency

Cause	Examples
Inadequate diet	Low income, elderly with limited function/income, alcoholics
Increased requirement	Diseases/conditions associated with rapid cell turnover (sickle cell anemia, thalassemia, leukemias, other malignancies, pregnancy, infancy)
Malabsorption	Ileitis, tropical sprue, nontropical sprue, blind loop syndrome
Drug inhibition	Oral contraceptives, long-term anticoagulant therapy, phenobarbital, primidone, phenytoin, antimetabolite chemotherapy
Biologic competition	Bacterial overgrowth in small intestine

alcoholic individuals, the ethanol appears to impair release of folate from the liver and can be toxic to erythroid precursors. Erythroid precursors in alcoholism are frequently vacuolated. In alcoholic individuals who have liver disease but have an adequate diet, the anemia is macrocytic but not megaloblastic.

Increased Requirement

In individuals with increased cell replication, the normal daily intake of folic acid may not be sufficient to maintain normal DNA synthesis. Without folate supplements, folate stores can be rapidly depleted. This occurs in hemolytic anemias such as sickle cell anemia and thalassemia, in myeloproliferative diseases such as leukemia, and in metastatic cancers. Anemia in pregnancy is common and can be caused by deficiencies of iron and/or folic acid. The deficiency of folic acid is related to the limited reserves of this nutrient and a 5–10-fold increased demand for its use created by the growing fetus. Prophylactic folic acid supplements are usually prescribed during pregnancy.[19]

Malabsorption

Intestinal diseases affecting the upper small intestine, which interfere with the absorption of nutrients, can cause a folate deficiency. The most common conditions of this type include ileitis, tropical sprue, and nontropical sprue. The blind loop syndrome associated with an overgrowth of bacteria can cause a folate deficiency because the bacteria preferentially utilize the folate.

Drug Inhibition

Megaloblastic anemia has also been associated with certain drugs including oral contraceptives, long-term anticoagulant drugs, phenobarbital, primidone, and phenytoin. Anemia occasionally is not present even though serum and erythrocyte folate are depressed.

☑ CHECKPOINT 15-6

What is the most common cause of folate deficiency, and in what groups of individuals is it usually found?

Laboratory Analysis of Folate Deficiency

Both serum and erythrocyte folate levels are decreased in folate deficiency. Serum folate reflects the folic acid intake over the last several days, whereas erythrocyte folate reflects the folate available when the red cell was maturing in the bone marrow and reflects the net folate level over the preceding several months. Serum folate can be falsely increased with even slight hemolysis of the sample. Low serum folate can indicate an imminent folic acid deficiency and precedes erythrocyte folate deficiency.[15]

Care must be taken in interpreting folate results because both serum and erythrocyte folate can be falsely increased or decreased in a variety of conditions (Table 15-5 ★). Neither serum (methyl-THF) nor erythrocyte folate (heterogeneous folate mixture with various polyglutamate chain lengths) is a good indicator of folate stores if there is a deficiency of cobalamin. A cobalamin deficiency leads to the accumulation of methyl-THF because the donation of the methyl group to homocysteine and the release of free THF is impaired. In addition, cobalamin is required for normal transfer of methyl-THF to the cells and for keeping the folate in the cell as conjugated folate. Thus, serum folate can be falsely increased (by 20–30%) and erythrocyte folate falsely decreased

★ **TABLE 15-5** Causes of False Increases and Decreases in Folate Levels

	Erythrocyte Folate	Serum Folate
False increase	Early folate deficiency	Recent increase in dietary folate
	Reticulocytosis	Hemolysis of blood sample
	Recent RBC transfusion	Coexisting cobalamin deficiency
False decrease	Cobalamin deficiency	Recent low dietary intake
	Recent alcohol consumption	Gallium or technetium administration

in cobalamin deficiency due to folate trapping as methyl-THF. Epithelial changes in the GI tract that accompany cobalamin deficiency can lead to malabsorption of folic acid in which case both serum and erythrocyte folate levels are decreased.

Competitive folate-binding assays (indirect immunoassays) using chemiluminescence methods have replaced microbiological assays for both serum and RBC folate.

Cobalamin (Vitamin B_{12})

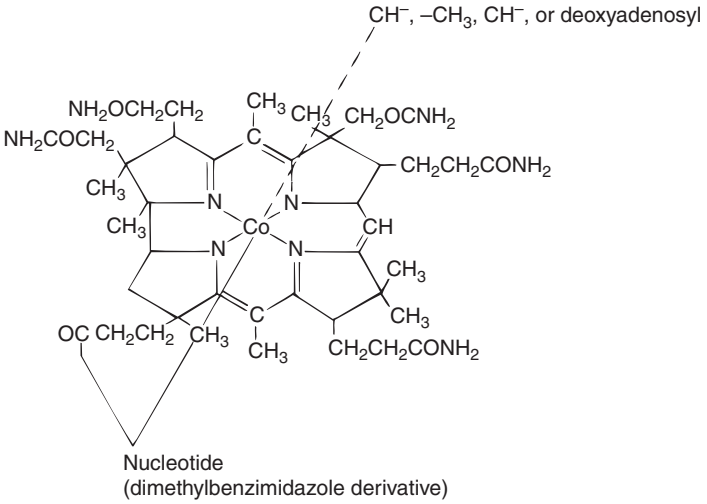
Cobalamin is required for DNA synthesis and neurologic function. Specific diagnosis of cobalamin deficiency is critical because associated neurologic damage can be irreversible. Pernicious anemia is a specific form of cobalamin deficiency and will be discussed in detail later in this chapter.

Structure and Function

Vitamin B_{12} is a commonly used generic term for a family of cobalamin vitamins in which ligands can be chelated to cobalt. The more accurate terminology when referring to this family of vitamins is *cobalamin*. Vitamin B_{12} refers specifically to the therapeutic form of crystalline cobalamin that contains the ligand cyanide (cyanocobalamin), a form not naturally found in the body but used for treating cobalamin deficiency. In hematology literature, the terms *cobalamin* and *vitamin B_{12}* are often used interchangably. In this text we will use the term *cobalamin*.

Cobalamin is structurally classified as a corrinoid, a family of compounds with a corrin ring. The molecule has three portions: (1) a corrin ring composed of four reduced pyrrole groups with a cobalt at the center (cobalamin gets it name from the central cobalt), (2) a nucleotide that lies almost perpendicular to the ring attached to the cobalt, and (3) various ligands (a β-group) attached to the cobalt on the opposite side of the ring from the nucleotide (Figure 15-8 ■). The β-group in cobalamin is cyanide, methyl, adenosyl, or hydroxyl. Adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) act as coenzymes in biological reactions. Hydroxycobalamin (OHCbl) and cyanocobalamin (CnCbl) are not metabolically active forms of cobalamin but can be converted to the active methyl and adenosyl forms by tissue enzymes.

As mentioned previously, methionine is formed from homocysteine when a methyl group is transferred from N^5-methyl THF to homocysteine in a cobalamin-dependent reaction catalyzed by methionine synthase (Figure 15-7a). For this reaction, the vitamin



■ **FIGURE 15-8** Molecular structure of the cobalamin molecule.

must be in the MeCbl form. A cobalamin deficiency traps newly acquired folate in the N^5-methyl THF form (the form in which the cell acquires folate from the plasma). N^5-methyl THF is not efficiently conjugated with glutamic acids and thus leaks back out of cells (see the discussion of folate metabolism). The result is an intracellular functional deficiency of folate and a block of DNA synthesis.

AdoCbl is required for only one mammalian reaction: the conversion of methylmalonyl CoA to succinyl CoA (Figure 15-7c). AdoCbl acts as a coenzyme with methylmalonyl CoA mutase in this reaction. Increased urinary excretion of methylmalonic acid (MMA), a precursor of methylmalonyl-CoA, is a diagnostic aid in cobalamin deficiency.

☑ CHECKPOINT 15-7

A patient has the following results: vitamin B_{12} 50 pg/mL, serum folate 4 ng/dL, RBC folate 100 ng/mL. Interpret these results.

Metabolism

Defects in any of the steps of cobalamin metabolism can lead to a cobalamin deficiency and megaloblastic anemia. The laboratory plays a critical role in both assessing the level of the vitamin and determining the cause of low levels. Thus, it is important to understand the metabolism of this important nutrient.

Absorption

Cobalamin is present in most foods of animal origin including milk, eggs, and meat. The vitamin complex is released from food by peptic digestion at the low pH in the stomach and binds tightly to a haptocorrin (HC)-like protein, a cobalamin binding protein secreted in the saliva and in the stomach. This binding protein was previously known as R-protein, named because of its rapid electrophoretic mobility compared with the mobility of intrinsic factor. HC is the preferred binding protein for cobalamin released from food. The HC–cobalamin complex protects the cobalamin from degradation by the hydrochloric acid in the stomach. In the duodenum, pancreatic proteases degrade HC, releasing cobalamin. The released cobalamin quickly binds to intrinsic factor (IF), which resists pancreatic degradation. Intrinsic factor is a glycoprotein secreted by parietal cells of the gastric mucosa in response to the presence of food, vagal stimulation, histamine, and gastrin. IF binds cobalamin with a 1:1 stoichiometry and is required for the intestinal absorption of cobalamin. The IF–cobalamin complex resists digestion and passes through the jejunum into the ileum where it binds the specific IF receptor (cubulin) on the microvilli of ileal mucosal cells[14] (Figure 15-9 ■). Binding requires a pH of 5.4 or higher as well as calcium. Following attachment of the IF–cobalamin complex to cubulin, the entire complex is taken into the mucosal cell by endocytosis. Cobalamin is released from IF and enters the portal blood while IF is degraded.[14] The IF-receptors are recycled to the microvilli of the ileum where they participate in absorption of more IF–cobalamin complexes. Large oral doses of cobalamin can be absorbed by simple diffusion without IF (see the section on "Therapy").

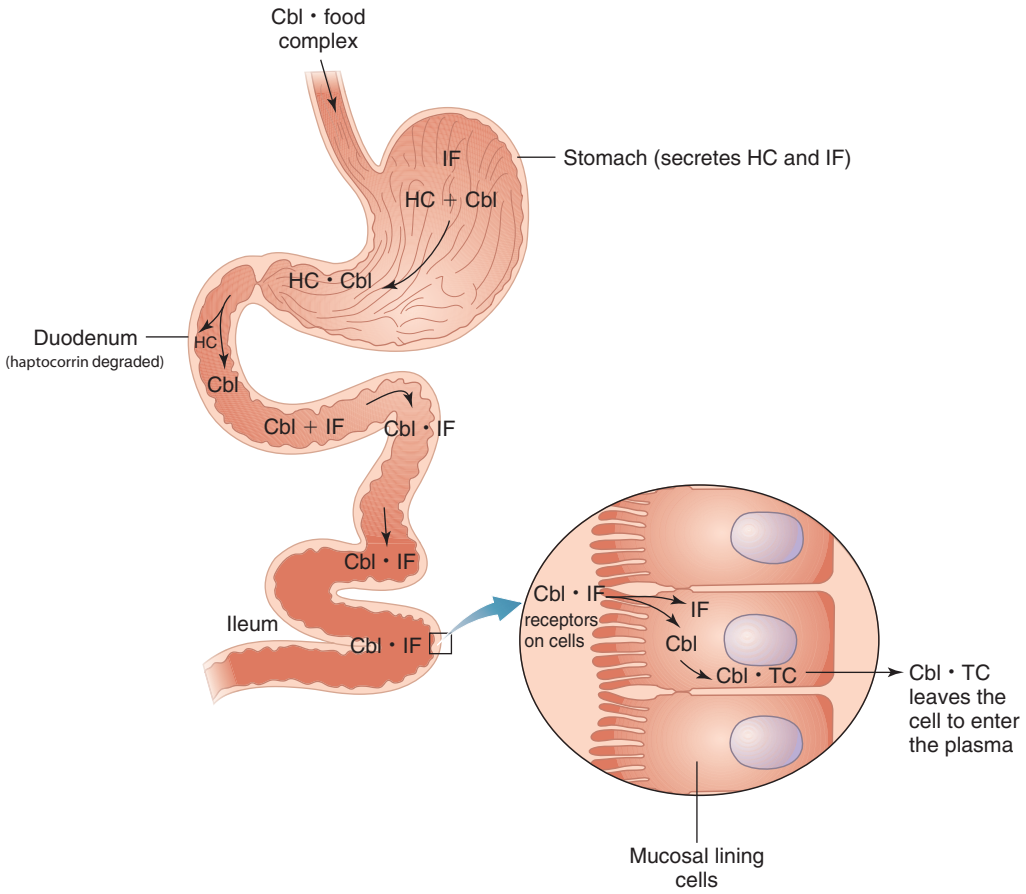
Transport

Proteins that transport cobalamin in the blood are transcobalamin (previously known as *transcobalamin II*) and haptocorrins (previously known as *transcobalamin I* and *transcobalamin III* or *R-proteins*). These plasma haptocorrins belong to the same family of proteins as the gastric haptocorrin.

Transcobalamin (TC) is produced in many types of cells, including hepatocytes, enterocytes, macrophages, and hematopoietic precursors in the bone marrow. Although TC carries only a small fraction of the total cobalamin in the plasma, it binds 90% of the newly absorbed cobalamin and is the primary plasma protein that mediates transfer of cobalamin into the tissues. TC also binds corrins that are chemically similar to cobalamin but have no known function in mammalians (cobalamin analogues). The TC–cobalamin complex is thought to be formed within the ileal mucosal cells and released to the blood.[14] This transport complex disappears rapidly from blood ($T_{1/2}$ of 6–9 minutes) as it is taken up by cells in the liver, bone marrow, and other dividing cells that have specific receptors for TC (TCblR, CD320). The TC–cobalamin complex is internalized by receptor-mediated endocytosis. Once inside the cell, cobalamin is released from TC and utilized, and the TC is degraded. Congenital deficiency of TC produces a severe megaloblastic anemia in infancy. However, serum cobalamin concentration in this condition is normal.

The functions of the haptocorrins, a group of immunologically related proteins, are less well understood. They are synthesized by the mucosal cells of the organs that secrete them and by phagocytes. They are found in body fluids including plasma, saliva, amniotic fluid, milk, and gastric juice. Haptocorrins (HC) bind 70–90% of circulating cobalamin forming the cobalamin–HC complex. Cobalamins undergo a significant amount of enterohepatic recirculation. The cobalamin–HC complex attaches to an asialoglycoprotein receptor on hepatocytes. Clearance of HC from plasma is much slower ($T_{1/2}$ 9–10 days) than that of TC. Within the hepatocyte HC is degraded and cobalamin is excreted into the bile where it binds to haptocorrin. This HC–cobalamin complex enters the intestine where the cobalamin is released from HC by pancreatic proteases and bound by IF in the same manner as dietary cobalamin.

HC binds ligands less specifically and more tightly than TC. It binds a wide variety of corrinoids in addition to cobalamin. These analogues are transported by HC to the liver, metabolized,



■ **FIGURE 15-9** Assimilation of cobalamin.
HC = haptocorrin; IF = intrinsic factor; TC = transcobalamin; Cbl = Cobalamin

and excreted in the bile. The analogues bind poorly to IF and therefore are not absorbed but are excreted in the feces. Thus, HC can serve to clear the body of nonphysiologic cobalamin analogues.

In plasma, HC is largely produced by granulocytes. Haptocorrins are increased in myeloproliferative disorders, presumably due to excess proliferation of granulocytes.

Requirements

About 3–5 mcg of cobalamin per day is needed to maintain normal biochemical functions. It is estimated that only about 70% of cobalamin intake is absorbed, which suggests that the diet should include 5–7 mcg of the vitamin per day. This amount is available in a regular "balanced" diet but not in a strict vegetarian diet. Cobalamin stores (~5000 mcg) are sufficient to provide the normal daily requirement for about 1000 days. Therefore, it takes several years to develop a deficiency if no cobalamin is absorbed from the diet. About half of the vitamin is stored in the liver, and the rest is located in the heart and kidneys.[20]

☑ CHECKPOINT 15-8

Explain why there is a megaloblastic anemia in transcobalamin deficiency when the serum vitamin B_{12} concentration is normal.

Pathophysiology of Cobalamin Deficiency

Deficiency of cobalamin is reflected by (1) impaired DNA synthesis (megaloblastic anemia) and (2) defective fatty acid degradation (neurologic symptoms).

Impaired DNA Synthesis

A deficiency in either cobalamin or folic acid results in impaired production of methylene-THF, a defect in thymidylate synthesis, and ultimately a defect in DNA synthesis (Figure 15-7a). This produces megaloblastic anemia and epithelial cell abnormalities. All dividing cells including the hematopoietic cells in the bone marrow are affected.

Defective Fatty Acid Degradation

AdoCbl is a cofactor in the conversion of methylmalonyl CoA to succinyl CoA. In cobalamin deficiency, there is a defect in degradation of propionyl CoA to methylmalonyl CoA and, finally, to succinyl CoA. As propionyl CoA accumulates, it is used as a primer for fatty acid synthesis, replacing the usual primer acetyl CoA. This results in fatty acids with an odd number of carbons. These odd-chain fatty acids are incorporated into neuronal membranes, causing disruption of membrane function. It is probable that **demyelination** (destruction, removal, or loss of the lipid substance that forms a myelin sheath around the axons of nerve fibers), a characteristic finding in cobalamin deficiency, is a result of this erroneous fatty acid synthesis.

A critical feature of demyelination in cobalamin deficiency is neurological disease. Peripheral nerves are most often affected,

presenting initially as motor and sensory neuropathy. The brain and spinal cord can also be affected leading to dementia, spastic paralysis, and other serious neurological disturbances. Neurologic damage has been known to occur occasionally without any sign of anemia or macrocytosis, making accurate diagnosis difficult but critical.[21] The bone marrow, however, always shows megaloblastic hematopoiesis. Neurological disease might not be totally reversible but, if treated early, can be partially resolved. Neurological disease does not occur in folate deficiency. Administration of synthetic folic acid corrects the anemia of cobalamin deficiency but does not halt or reverse neurological disease because synthetic folic acid, unlike dietary folate, is reduced directly to THF without the requirement of cobalamin as a cofactor. The THF can correct the megaloblastosis, but because there is a bypass of the cobalamin-dependent reaction of conversion of homocysteine to methionine, SAM—a metabolite considered critical to nervous system function—is not formed. Thus, it is essential to differentiate folate deficiency and cobalamin deficiency so that appropriate treatment can be given.

Gastritis and abnormalities of the gastrointestinal epithelium secondary to cobalamin deficiency can interfere with the absorption of folic acid and iron, complicating the anemia.

☑ CHECKPOINT 15-9

Explain why severe cobalamin deficiency sometimes presents with neurological disease.

Causes of Cobalamin Deficiency

Cobalamin deficiency has many causes, including lack of intrinsic factor (pernicious anemia), malabsorption, nutritional deficiency, and impaired utilization by tissues due to defective or absent transport proteins or enzymes (Table 15-6 ★).

Pernicious Anemia

Pernicious anemia (PA) is a specific term used to define the megaloblastic anemia caused by an absence of IF secondary to gastric atrophy. An absence of IF leads to cobalamin deficiency because the vitamin cannot be absorbed in its absence. PA is the most common cause of cobalamin deficiency, accounting for 85% of all deficiencies. Atrophy of gastric parietal cells is demonstrated by finding **achlorhydria** of gastric juice after histamine stimulation (these cells produce HCl as well as IF). PA is generally a disease of older adults, usually occurring after 40 years of age. This anemia is seen more commonly among people of Northern European background, especially Great Britain and Scandinavia, but can be found in all ethnic groups. More women than men are affected, and some patients have prematurely graying or whitening hair. Although no particular genetic abnormality has been identified, a positive family history of PA increases the risk of developing it by 20-fold. The incidence of gastric carcinoma in patients with PA is increased.[22,23]

PA is an autoimmune disease. The gastric atrophy is thought to result from immune destruction of the acid-secreting portion of the gastric mucosa.[14] Up to 90% of PA patients have antibodies against parietal cells.[24] However, these antibodies are not specific for pernicious anemia and are also found in patients with gastritis, thyroid disease, and Addison's disease. On the other hand, serum antibodies against intrinsic factor are found in ~75% of PA patients and are highly specific for PA. These IF antibodies are of two types: blocking and binding. Type I, or blocking antibodies, are antibodies to IF and prevent formation of the IF–cobalamin complex. Type II, or binding antibodies, are directed against the IF–cobalamin complex and prevent the IF–cobalamin complex from binding to ileal receptors. Binding antibodies are found in about half the sera that contain blocking antibodies. A number of findings suggest that the immune destruction of the gastric mucosa is not antibody mediated but more likely T-cell mediated. Patients with agammaglobulinemia have a higher than expected incidence of PA. Also, lymphocytes from PA patients have been shown to be hyperresponsive to gastric antigens.[14]

Pernicious anemia frequently occurs with other autoimmune diseases such as Graves disease and Hashimoto's thyroiditis, type I diabetes, and Addison's disease. In addition, a predisposition to PA can be inherited. Relatives of patients with PA have a higher incidence of antiparietal cell antibodies and anti-intrinsic factor antibodies than the general population, even in the absence of overt PA.[14]

Juvenile pernicious anemia is rare in children. It can occur secondary to a variety of conditions, including a congenital deficiency or abnormality of IF (the more common type, with lack of IF but otherwise normal gastric secretion), or more rarely, true PA of childhood. True PA of childhood has an absence of intrinsic factor, gastric atrophy, decreased gastric secretion, and antibodies against IF and parietal cells. Megaloblastic anemia in childhood from malabsorption of cobalamin can also be due to a congenital deficiency of TC, a congenital gastric haptocorrin deficiency, or selective malabsorption of cobalamin (Imerslund-Gräsbeck disease). The latter can be due to abnormal cubulin receptors in the ileum.

★ **TABLE 15-6** Causes of Cobalamin Deficiency and Associated Conditions

Cause	Associated Conditions
Malabsorption	Pernicious anemia (lack of IF)
	Gastrectomy or gastric bypass
	Crohn's disease
	Tropical sprue
	Celiac disease
	Surgical resection of the ileum
	Imerslund-Grääsbeck disease
	Pancreatic insufficiency
	Drugs (colchicine, neomycin, p-aminosalicylic acid, or omeprazole)
	Blind loop syndrome
	Diverticulitis
Biologic competition	Intestinal parasite (i.e., *Diphyllobothrium latum*)
	Leishmaniasis
	Bacterial overgrowth
Nutritional deficiency	Strict vegetarian diets
	Pregnant women on a poor diet
	Malnutrition
Impaired utilization	Transcobalamin deficiency
	Nitrous oxide inhalation

Other Causes of Malabsorption

Pernicious anemia is only one specific cause of cobalamin malabsorption, which also can be caused by a loss of IF secondary to gastrectomy or secondary to diseases that prevent binding of the IF–cobalamin complex in the ileum. An iron deficiency usually precedes cobalamin deficiency in patients who have had a gastrectomy. Diseases that can affect the absorption of the IF–cobalamin complex in the ileum include Crohn's disease, tropical sprue, celiac disease, and surgical resection of the ileum. In Imerslund-Gräsbeck disease, the IF receptors (cubulin) are missing or abnormal, causing a form of juvenile megaloblastic anemia. Patients with severe pancreatic insufficiency experience a lack of absorption of cobalamin because the vitamin cannot be released from HC and transferred to IF. Normally, pancreatic proteases are responsible for degrading HC. Certain medications can interfere with intestinal absorption. In addition, conditions that allow a buildup of bacteria in the small bowel can cause a cobalamin deficiency as the bacteria preferentially take up the vitamin before it reaches the ileum. This situation occurs in the blind loop syndrome and in diverticulitis. Infestation with the fish tapeworm *Diphyllobothrium latum* can cause a deficiency as the worm accumulates the vitamin avidly.

Nutritional Deficiency

Dietary deficiency of cobalamin is rare in the United States. Food from animal sources, especially liver, is rich in cobalamin. Strict vegetarian diets, however, do not supply cobalamin and individuals following these diets can develop a deficiency over a period of years. Occasionally, pregnant women with a poor diet can develop a deficiency presumably due to an increased demand by the developing fetus. However, folic acid deficiency is a more common cause of megaloblastic anemia in pregnancy due to the lower preexisting stores of this nutrient.

Other Causes

Transcobalamin deficiency produces megaloblastic anemia because it is the major transport protein responsible for delivering the vitamin to the tissues. In TC deficiency, cobalamin is absorbed normally, and serum cobalamin levels are normal because of the relatively high amount bound to HC. Tissue cobalamin deficiency, including megaloblastic anemia, develops, however, because the cellular receptors recognize only TC-bound, not the haptocorrin-bound cobalamin.[14]

Nitrous oxide, N_2O ("laughing gas"), abuse has been reported to result in a cobalamin deficiency and megaloblastic anemias. N_2O rapidly inactivates methionine synthetase, for which cobalamin is a coenzyme. Cobalamin cleaves N_2O and at the same time is oxidized to an inert form. This leads to a rapid deficiency.

☑ CHECKPOINT 15-10

Why is pernicious anemia considered an autoimmune disorder?

Laboratory Analysis of Cobalamin Deficiency

Laboratory diagnosis of pernicious anemia and/or cobalamin deficiency usually begins with a serum cobalamin assay. If the cobalamin level is decreased and there are clinical symptoms of a deficiency, no further testing is warranted before therapy is initiated.[25] However, studies have shown that serum cobalamin does not always detect a cobalamin deficiency, and further testing could be necessary if a deficiency is suspected. Furthermore, serum cobalamin can appear falsely decreased or falsely normal/increased in some conditions (Table 15-7 ★). Cobalamin deficiency can be masked by folate therapy.

★ **TABLE 15-7** Causes of False Decrease and False Normal/Increase in Serum Cobalamin Levels

False Decrease	False Normal/Increase
Folate deficiency	Cobalamin treatment
Pregnancy (last trimester)	Liver disease
Oral contraceptive use	Nitrous oxide exposure
Multiple myeloma	Chronic myelogenous leukemia
Elderly	Polycythemia vera
Haptocorrin deficiency	Transcobalamin deficiency

A specific test that measures the increased excretion of methylmalonic acid (MMA) in the urine indirectly indicates a decrease in cobalamin concentration. Up to 40% of patients may have increased MMA levels in the urine but normal cobalamin levels.[22] These patients, however, show laboratory and clinical evidence of cobalamin deficiency. The only condition in which MMA is increased in addition to cobalamin deficiency is congenital methylmalonic aciduria. This condition is caused by complete or partial deficiencies of enzymes and cofactors involved in the conversion of methlmalonyl CoA to succinyl CoA. There is no associated hyperhomocysteinemia or megaloblastic anemia. Determination of MMA concentration also is useful in distinguishing a cobalamin deficiency and folate deficiency.

Homocysteine is increased in the plasma of patients with cobalamin or folate deficiency.[26] Monitoring serum levels can be an early detector of cobalamin deficiency, and recent studies have concluded that MMA and homocysteine are the most sensitive and specific indicators of deficiency.[26–29] Some studies have shown that in addition to being a good indicator of cobalamin deficiency, increased homocysteine levels are associated with a three-fold increase risk in myocardial infarction as well as venous thrombosis.[28] Increased concentrations of both analytes can be found in many patients with normal serum cobalamin concentrations.[28] Some clinicians recommend initial testing for serum cobalamin levels and following up low or borderline normal results with MMA and homocysteine measurements.[29] Others have found that MMA and homocysteine measurements are more sensitive tests of early pernicious anemia, preceding hematologic abnormalities and decreased serum cobalamin levels, and recommend them as a superior testing regimen (Figure 15-7 ■).

Homocysteine and MMA tests also are helpful in determining a patient's response to treatment with cobalamin and folate. The serum MMA level remains increased in cobalamin-deficient patients treated inappropriately with folate. Folate-deficient patients treated inappropriately with cobalamin have increased serum homocysteine level. See Table 15-8 ★ for a list of the advantages and disadvantages of laboratory tests used to diagnose cobalamin deficiency.[6]

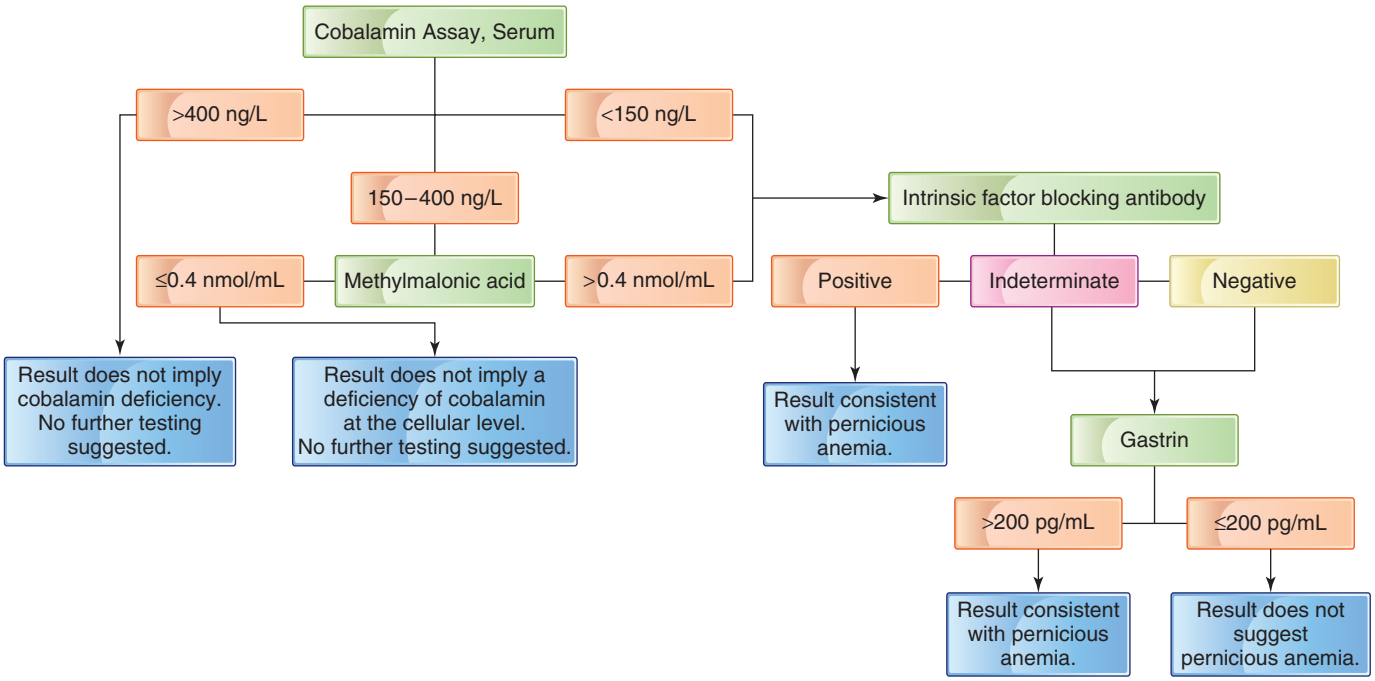
The serum cobalamin and MMA assays establish the existence of a cobalamin deficiency but do not provide a distinction between PA and other causes of cobalamin deficiency. Gastric analysis and the

★ **TABLE 15-8** Tests Used to Diagnose Cobalamin Deficiency[a]

Test	Advantages/Disadvantages
Cobalamin/serum	High sensitivity; widely available; variation in reference intervals according to method of analysis; measures both HC and TC although only holo-TC is available for cells; falsely decreased in folate and HC deficiency
Methylmalonic acid (MMA)/urine	High sensitivity and specificity; increased concentration helps differentiate cobalamin from folate deficiency; increased level may precede hematologic abnormalities; helpful in determining patient response to cobalamin treatment; falsely increased in reduced renal function; not readily available; expensive
Homocysteine/plasma	High sensitivity; low specificity; increased level may precede hematologic abnormalities; helpful in determining patient response to cobalamin treatment; does not differentiate cobalamin deficiency from folate deficiency; falsely increased in reduced renal function, hypothyroidism, hypovolemia
Holo-TC	Sensitivity and specificity similar to cobalamin; measure of cobalamin available to tissue; falsely increased in liver disease and in reduced renal function; complicated interpretation (see section Laboratory Analysis of Cobalamin Deficiency"); not a readily available test
Complete blood count with differential	High sensitivity of macrocytosis with megaloblastic changes; specificity of macrocytosis without megaloblastic changes is low; not readily recognizable in subclinical cobalamin deficiency

[a] Sensitivity and specificity of these tests may be lower in subclinical cobalamin deficiency; subclinical deficiency has no known clinical expression and diagnosis depends on biochemical markers; recommended diagnostic test combination for cobalamin deficiency is serum cobalamin and MMA. Both should be abnormal for a diagnosis of cobalamin deficiency.
Holo-HC = Transcobalamin II with cobalamin attached

Schilling test are more useful in establishing the specific diagnosis of PA but are rarely performed anymore. A recommended testing algorithm for laboratory diagnosis of PA is shown in Figure 15-10 ■.[30] The order of testing can improve the efficiency of proper diagnosis by first measuring serum cobalamin and MMA. If serum cobalamin is borderline or low and MMA is increased, the diagnosis of PA should be confirmed with testing for intrinsic factor blocking antibody (IFBA) and serum gastrin levels. The serum gastrin test may be used to replace the Schilling test (see the following discussion). Gastrin is usually markedly increased in PA due to gastric atrophy. In contrast



■ **FIGURE 15-10** Testing algorithm for the diagnosis of pernicious anemia.

to the Schilling test, the gastrin test does not require radioisotopes and is less expensive.

Measurement of holo-transcobalamin (holo-TC), the fraction of plasma cobalamin bound to TC, can be an early marker for cobalamin deficiency. Because cellular receptors specifically mediate the uptake of holo-TC but not haptocorrin-bound cobalamin (holo-HC), holo-TC could provide a more accurate view of patients' cobalamin status than total cobalamin levels that include both holo-TC and holo-HC.[31] Low holo-TC levels, however, may only be temporary or may even remain at the subclinical level rather than progress to pathologic levels. The interpretation of holo-TC values is complicated by the fact that it is unclear whether low results reflect insufficient availability to tissues or some impairment in the ability of the holo-TC complex to leave the ileal mucosal cells.[32] Previously tested by enzyme-linked immunosorbent and radioimmunoassay techniques, holo-TC is now available as an automated monoclonal antibody assay with a reference interval of approximately 20–125 pmol/L.[33]

Gastric Analysis

Because atrophy of the parietal cells is a universal feature of PA, an absence of free HCl in gastric juice after histamine stimulation is indicative of PA. Parietal cells secrete both HCl and intrinsic factor; thus an absence of HCl is indirect evidence for lack of IF. After histamine stimulation in patients with pernicious anemia, the pH fails to fall below 3.5, and gastric volume, pepsin, and rennin are decreased. Approximately 80% of cases of PA have increased gastrin. Testing for anti-intrinsic factor antibodies, serum gastrin, and serum pepsinogin A and C together as a group provides a sensitive indication of gastric atrophy and pernicious anemia.[33]

Schilling Test

The **Schilling test** is a definitive test useful in distinguishing cobalamin deficiency due to malabsorption, dietary deficiency, or absence of IF. The test measures the amount of an oral dose of radioactively labeled crystalline vitamin B_{12} that is absorbed in the gut and excreted in the urine. The patient is given a dose of ^{57}Co-labeled B_{12} orally with or followed within two hours by an intramuscular injection of unlabeled vitamin B_{12}. The injection is termed the *flushing dose*; its purpose is to saturate all cobalamin receptors in the tissues. Thus, any of the labeled oral dose absorbed in the gut and passing into the blood will be in excess of available receptor uptake. The excess is filtered by the kidney and appears in the urine. Urine is collected for 24 hours, and its radioactivity is determined. If >7.5% of the standard oral dose is excreted, absorption is said to be normal. Excretion in PA and in malabsorption syndromes is <7.5% because the labeled oral vitamin B_{12} is not absorbed.

If excretion is <7.5%, part II of the Schilling test is performed to distinguish between PA and other causes of malabsorption. In part II, the oral dose of labeled B_{12} is accompanied by a dose of intrinsic factor. The remainder of the test is the same as in part I. If part II shows >7.5% excretion, absorption is considered normal with the lack of absorption in part I due to the lack of IF. The diagnosis is PA. If part II is abnormal, the patient likely has another malabsorption defect such as sprue (Table 15-9 ★).

Several points must be considered when interpreting the results of a Schilling test. First, the test results are not valid with the presence of renal disease. The patient may have been able to absorb the vitamin but cannot filter the excess vitamin efficiently because of abnormal kidney function. Second, incomplete collection of urine invalidates the results. Incontinence or inability to empty the bladder gives false low values even when absorption was normal. Spuriously low urinary excretion in part II can also be due to the inability to absorb IF-B_{12} because of megaloblastoid epithelial changes in the gut.

The Schilling test is seldom used now because of the difficulties in using radioisotopes and the inconvenience of the test for the patient. An additional problem is that the absorption of crystalline vitamin B_{12} used in the oral dose can differ from the absorption of cobalamin in food.[15,34] For these reasons, MMA and homocysteine are better markers for early detection of PA or cobalamin deficiency.

On the other hand, the Schilling test has advantages. First, the test results can suggest what further testing (e.g., stool analysis for *D. latum*) should be done. Second, results can help identify how to treat the clinical condition and the duration of treatment.[35]

★ **TABLE 15-9** Conditions Associated with Abnormal Schilling Test Results

Corrected by Intrinsic Factor in Part II	Not Corrected by Intrinsic Factor in Part II
Pernicious anemia	Crohn's disease
Abnormal intrinsic factor molecule	Tropical sprue
	Celiac disease
Hereditary intrinsic factor deficiency	Surgical resection of the ileum
	Imerslund-Gräsbeck disease
Gastrectomy	Pancreatic insufficiency
	Drugs
	Blind loop syndrome
	Diverticulitis
	Intestinal parasite— *Diphyllobothrium latum*
	Transcobalamin deficiency

CASE STUDY *(continued from page 282)*

A Schilling test and antibody testing had the following results:

Schilling test	Part I, without intrinsic factor, 1% Part II, with intrinsic factor, 8%
Intrinsic-factor-blocking antibodies	Positive to a titer of 1:6400

5. What is this patient's definitive diagnosis?

CobaSorb Test

The CobaSorb test is an alternative test designed to measure the absorption of cobalamin.[6,36] There are three parts to this test in which the patient is given nonradioactive cobalamin either alone or with recombinant IF or haptocorrin. Blood levels of holo-TC and holo-HC are measured before and after administration of the cobalamin. An increase in the concentrations of these plasma transport proteins saturated with cobalamin after administration of the cobalamin is reflective of active cobalamin absorption. Refer to Table 15-10 ★ for a list of tests used to investigate the cause of a cobalamin deficiency.

★ **TABLE 15-10** Tests Used to Investigate the Cause of Cobalamin Deficiency[a]

Test	Advantages/Disadvantages
Schilling test	Gold standard for measure of cobalamin absorption; high specificity for PA; requires handling and administration of radioactive cobalamin; requires collection of a 24 hour urine sample; false positive with reduced renal function; decreased availability of the test
IF blocking antibodies	High specificity for lack of IF; low sensitivity; pathognomonic for PA
Pepsinogen and gastrin	High sensitivity; low specificity for PA; mirrors gastric function; increased in gastric atrophy
Parietal cell antibodies	May be present in PA; low specificity
CobaSorb	Alternative to Schilling test; reflects absorption of cobalamin; use in routine practice needs further evaluation

[a]These tests are used primarily to evaluate cobalamin deficiency due to pernicious anemia. Additional testing may be necessary to evaluate other causes of cobalamin deficiency. Patient history is also important in diagnosis.
IF = intrinsic factor; PA = pernicious anemia; holo-TC = TCII with attached cobalamin

☑ CHECKPOINT 15-11

What two lab tests are the most specific indicators of cobalamin deficiency?

Therapy

Therapeutic trials in megaloblastic anemia using *physiologic* doses of either vitamin B_{12} or folic acid produce a reticulocyte response only if the specific vitamin that is deficient is being administered. For instance, small doses (1 mcg) of vitamin B_{12} given daily produce a reticulocyte response in cobalamin deficiency but not in folic acid deficiency. On the other hand, large therapeutic doses of cobalamin or folic acid can induce a partial response to the other vitamin deficiency as well as the specific deficiency.

Generally, it is best to determine which deficiency exists and to treat the patient with the specific deficient vitamin. Large doses of folic acid will correct the anemia in cobalamin deficiency but do not correct or halt demyelination and neurologic disease. This makes diagnosis and specific therapy in cobalamin deficiency critical. Specific therapy causes a rise in the reticulocyte count after the fourth day of therapy. Reticulocytosis peaks at about 5–8 days and returns to normal after 2 weeks. The degree of reticulocytosis is proportional to the severity of the anemia with more striking reticulocytosis in patients with severe anemia. The hemoglobin rises about 2–3 g/dL every 2 weeks until normal levels are reached. The marrow responds quickly to therapy, as evidenced by pronormoblasts (normal) appearing within 4–6 hours and nearly complete recovery of erythroid morphologic abnormalities within 2–4 days. Granulocyte abnormalities disappear more slowly. Hypersegmented neutrophils can usually be found for 12–14 days after therapy begins.

Specific therapy can reverse the peripheral neuropathy of cobalamin deficiency, but spinal cord damage is usually irreversible. Pernicious anemia must be treated with lifelong monthly parenteral doses of hydroxycobalamin (OHCbl) because of these patients' inability to absorb oral cobalamin. Recently, it was reported that large doses of cobalamin therapy (usually 1000–2000 mcg/day) administered orally could be feasible if the patient is followed carefully.[24] The oral treatment can be better tolerated and less expensive.[37] The rationale behind oral therapy using large doses of vitamin is that a small amount (from 1 to 3%) of the vitamin is absorbed by diffusion without IF.

CASE STUDY *(continued from page 293)*

6. How would the diagnosis change if the special testing results were as follows?

Schilling test	Part I, without intrinsic factor, 1% Part II, with intrinsic factor, 3%
Intrinsic-factor-blocking antibodies	Negative

7. What would you predict this patient's reticulocyte count to be?

Other Megaloblastic Anemias

A megaloblastic anemia occasionally is associated with drugs, congenital enzyme deficiencies, or other hematopoietic diseases.

Drugs

A large number of drugs that act as metabolic inhibitors can cause megaloblastosis (Table 15-11 ★). Some of these drugs are used in chemotherapy for malignancy. Although aimed at eliminating rapidly

proliferating malignant cells, these drugs are not selective. Any normal proliferating cells, including hematopoietic cells, are also affected.

Enzyme Deficiencies

Methionine synthase reductase (MSR) deficiency is a rare autosomal recessive disorder. A deficiency of this enzyme leads to a dysfunction of folate/cobalamin metabolism and results in hyperhomocysteinemia, hypomethioninemia, and megaloblastic anemia.[38] MSR is necessary for the reductive activation of methionine synthase and the resultant folate-cobalamin-dependent conversion of homocysteine to methionine.

Congenital Deficiencies

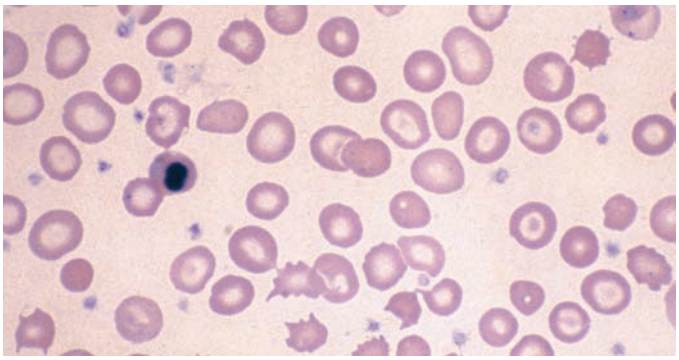
A congenital deficiency is a physiological aberration present at birth. The following congenital deficiencies result in megaloblastosis.

Orotic Aciduria

Inborn defects in enzymes required for pyrimidine synthesis or folate metabolism can result in megaloblastic anemia. *Orotic aciduria* is a rare autosomal recessive disorder in which there is a failure to convert orotic acid to uridylic acid. The result is excessive excretion of orotic acid. Children with this disorder also fail to grow and develop normally. The condition responds to treatment with oral uridine.

Congenital Dyserythropoietic Anemia

Congenital dyserythropoietic anemia (CDA) is actually a heterogeneous group of refractory, congenital anemias characterized by both abnormal erythropoiesis and ineffective erythropoiesis (Table 15-12 ★). There are three types: CDA I, CDA II, and CDA III. Types I and II are inherited as autosomal recessive disorders, and Type III is inherited in an autosomal dominant fashion. Red cell multinuclearity in the bone marrow and secondary siderosis are recognized in all types; however, megaloblastic erythroid precursors are present only in Type I and Type III.



■ **FIGURE 15-11** Peripheral blood from a case of congenital dyserythropoietic anemia type II (CDA-II). There is anisocytosis with microcytic, hypochromic cells as well as macrocytes and normocytes. The nucleated cell is an orthochromic normoblast showing lobulation of the nucleus (Wright-Giemsa stain; 1000× magnification).

- **CDA I** Bone marrow erythroblasts are megaloblastic and often binucleate with incomplete division of nuclear segments. The incomplete nuclear division is characterized by internuclear chromatin bridges.
- **CDA II** Bone marrow precursors are not megaloblastic but are typically multinucleated with up to seven nuclei (Figure 15-11 ■). Type II is distinguished by a positive acidified serum test (Ham test) but a negative sucrose hemolysis test. In the Ham test, only about 30% of normal sera are effective in lysing CDA II cells. This type has also been termed *hereditary erythroblastic multinuclearity with positive acidified serum test (HEMPAS)*. CDA II is the most common of the three types of CDA.
- **CDA III** This type of CDA is morphologically distinct from Types I and II because of the presence of giant erythroblasts (up to 50 mcM) containing up to 16 nuclei. Sometimes the erythrocytes are agglutinated by anti-I and anti-i antibodies.

★ **TABLE 15-11** Drugs That Can Cause Megaloblastosis

DNA Base Inhibitors			
Pyrimidine	**Purine**	**Antimetabolites**	**Other**
Azauridine	Acyclovir	Cytosine arabinoside	Azacytidine
	Adenosine arabinoside	Fluorocytidine	Cyclophosphamide
	Azathioprine	Fluorouracil	Zidovudine (AZT)
	Gancyclovir	Hydroxyurea	
	Mercaptopurine	Methotrexate	
	Thioguanine		
	Vidarabine		

★ **TABLE 15-12** Comparison of Congenital Dyserythropoietic Anemia (CDA) Types

Characteristics	CDA I	CDA II	CDA III
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal dominant
RBC multinuclearity	Present	Present	Present
Number of nuclei	2	Up to 7	Up to 16
Siderosis	Present	Present	Present
Megaloblastosis	Present	Absent	Present
Other characteristics	Incomplete nuclear division	Positive Ham test (HEMPAS)	RBC agglutination by anti-I and anti-i antibodies

Other Hematopoietic Diseases

The myelodysplastic syndromes are a group of stem cell disorders characterized by peripheral blood cytopenias and dyshematopoiesis. Erythroid precursors in the bone marrow frequently exhibit megaloblastic-like changes. There is occasionally a nonmegaloblastic macrocytic anemia. These diseases will be discussed in Chapter 25.

☑ CHECKPOINT 15-12

Which clinical type of CDA gives a positive Ham test result and presents with a normoblastic marrow?

MACROCYTIC ANEMIA WITHOUT MEGALOBLASTOSIS

The typical findings of megaloblastic anemia are not evident in other macrocytic anemias. The macrocytes in macrocytic anemias without megaloblastosis are usually not as pronounced and are usually round rather than oval as seen in megaloblastic anemia (Figure 15-1a, b). Hypersegmented neutrophils are not present, and leukocytes and platelets are quantitatively normal. Jaundice, glossitis, and neuropathy, the typical clinical findings in megaloblastosis, are absent. The cause of the macrocytosis without megaloblastosis is unknown in many cases. In some cases the macrocytes can be due to an increase in membrane lipids or to a delay in erythroblast maturation. Some diseases associated with nonmegaloblastic macrocytic anemia are listed in Table 15-13 ★. Three of the most common—alcoholism, liver disease, and reticulocytosis (stimulated erythropoiesis)—are discussed in this section.

★ **TABLE 15-13** Conditions Associated with Nonmegaloblastic Macrocytosis

Condition	Cause of Macrocytosis
Alcoholism	Direct toxic effect of alcohol on erythroid precursors
	Reticulocytosis associated with hemolysis or GI bleeding
	Liver disease: Abnormal RBC membrane lipid composition
	Can also be megaloblastic from folate deficiency
Liver disease	Increased RBC membrane lipids
Hemolysis or posthemorrhagic anemia	Reticulocytosis associated with stimulated erythropoiesis
Hypothyroidism	Unknown
Aplastic anemia	Unknown
Artifactual	Cold agglutinin disease
	Severe hyperglycemia
	RBC clumping
	Swelling of RBCs

Alcoholism

Alcohol abuse is one of the most common causes of nonanemic macrocytosis. It has been suggested that all patients with macrocytosis should be questioned about their alcohol consumption.[39] Macrocytosis associated with alcoholism is usually multifactorial and can be megaloblastic. Macrocytosis is probably the result of one or more of four causes: (l) folate deficiency due to decreased dietary intake, (2) reticulocytosis associated with hemolysis or gastrointestinal bleeding, (3) associated liver disease, and (4) alcohol toxicity.

Folate deficiency associated with a megaloblastic anemia is the most common cause of the macrocytosis found in hospitalized alcoholic patients. The deficiency probably results from poor dietary habits, although ethanol also appears to interfere with folate metabolism.

The reduced erythrocyte survival with a corresponding reticulocytosis has been associated with chronic gastrointestinal bleeding secondary to hepatic dysfunction (decreased coagulation proteins) or thrombocytopenia, hypersplenism from increased portal and splenic vein pressure, pooling of cells in splenomegaly, and altered erythrocyte membranes caused by abnormal blood lipid content in liver disease (Chapter 17). Stomatocytes are associated with acute alcoholism, but there appears to be no abnormal cation permeability, and hemolysis of these cells is not significant.

Liver disease is common in alcoholic individuals; typical hematologic findings associated with this disease are discussed in the following section. Even when anemia is absent, most alcoholic individuals have a mild macrocytosis (100–110 fL) unrelated to liver disease or folate deficiency. This can be caused by a direct toxic effect of ethanol on developing erythroblasts. Vacuolization of red cell precursors, similar to that seen in patients taking chloramphenicol, is a common finding after prolonged alcohol ingestion. If alcohol intake is eliminated, the cells gradually assume their normal size, and the bone marrow changes disappear. The association of a sideroblastic anemia and alcoholism is discussed in Chapter 12.

The multiple pathologies of this type of anemia result in the possibility of a variety of abnormal hematologic findings. Thus, it is possible to have a blood picture resembling that of megaloblastic anemia, chronic hemolysis, chronic or acute blood loss, liver disease, or (more than likely) a combination of these conditions. Alcohol can also cause disordered heme synthesis as discussed in Chapter 12.

Liver Disease

The most common condition associated with a nonmegaloblastic macrocytic anemia is liver disease (including alcoholic cirrhosis). The causes of this anemia are multifactorial and include hemolysis, impaired bone marrow response, folate deficiency, and blood loss (Table 15-14 ★). Although macrocytic anemia is the most common form of anemia in liver disease, occurring in >50% of the patients, normocytic or microcytic anemia can also be found depending on the predominant pathologic mechanism.

Erythrocyte survival appears to be significantly shortened in alcoholic liver disease, infectious hepatitis, biliary cirrhosis, and obstructive jaundice. The reason for this is unknown. Cross-transfusion studies in which patient cells are infused into normal individuals demonstrate an increase in patient cell survival. This suggests that an extracellular factor is probably responsible for cell hemolysis. The spleen is thought to play an important role in sequestration and hemolysis in individuals

★ **TABLE 15-14** Causes and Characteristics of Anemia in Liver Disease

Causes	Anemia Type	Characteristics
Abnormal liver function	Macrocytic	MCV normal to increased
		Increased RBC membrane cholesterol resulting in target cells and acanthocytes
		Normal to slightly increased reticulocytes
Folic acid deficiency	Macrocytic	MCV increased
		Pancytopenia
		Ovalocytes and teardrop cells
		Normal to decreased reticulocytes
		Functional folate deficiency
Hemolysis	Normocytic or macrocytic	MCV normal to increased
		Spur cells and schistocytes
		Increased reticulocytes
Hypersplenism	Normocytic	MCV normal
		Pancytopenia
		Increased reticulocytes
		Portal hypertension
Marrow hypoproliferation	Normocytic	MCV normal
		Pancytopenia
		Decreased reticulocytes
		Associated with renal disease or alcohol suppression
Chronic blood loss	Microcytic	MCV decreased
		Iron deficiency
		Leukocyte and platelet counts slightly increased
		Reticulocytes increased
		Commonly associated with gastrointestinal bleeding

with splenomegaly or hypersplenism. In some cases, hemolysis is well compensated for by an increase in erythropoiesis and there is no anemia. In some patients with alcoholic liver disease, a heavy drinking spree produces a brisk but transient hemolysis. These patients also show abnormal liver function and have markedly increased levels of plasma triglycerides.

Abnormalities in erythrocyte membrane lipid composition are common in hepatitis, cirrhosis, and obstructive jaundice. Both cholesterol and phospholipid are increased, resulting in cells with an increased surface area–to–volume ratio. This abnormality is not thought to cause decreased cell survival. In contrast, in severe hepatocellular disease, erythrocyte membranes have an excess of cholesterol relative to phospholipid, which decreases the erythrocyte deformability. This membrane lipid imbalance is associated with the formation of spur cells in which the erythrocyte exhibits spikelike projections. These cells have a pronounced shortened life span leading to an anemia termed *spur cell anemia* (Chapter 17).

Kinetic iron studies have revealed that the bone marrow response in liver disease can be impaired. It has been proposed that liver disease can affect the production of erythropoietin because this organ has been shown to be an important extrarenal source of the hormone.[40] In alcoholic cirrhosis, the alcohol may have a direct suppressive effect on the bone marrow.

Clinical findings and symptoms in liver disease are secondary to the abnormalities in liver function. The liver is involved in many essential metabolic reactions and in the synthesis of many proteins and lipids. Therefore, the anemia is a minor finding among the abnormalities associated with this organ's dysfunction.

The anemia is usually mild with an average hemoglobin concentration of about 12 g/dL. With complications, the anemia can be severe. The erythrocytes can appear normocytic, macrocytic (usually not >115 fL MCV), or microcytic. A discrepancy between the MCV and the appearance of the cells microscopically often occurs. In these cases, thin, round macrocytes (as determined by diameter) with target cell formation are found on the blood smear, but the MCV is within normal limits. The reticulocyte count can be increased, but the RPI is usually <2 unless hemolysis is a significant factor. Thrombocytopenia is a frequent finding, and platelet function can be abnormal. Various nonspecific leukocyte abnormalities have been described including neutropenia, neutrophilia, and lymphopenia. The bone marrow is either normocellular or hypercellular, often with erythroid hyperplasia. The precursors are qualitatively normal unless folic acid deficiency is present. In this case, megaloblastosis is apparent with the typical associated blood abnormalities.

Other laboratory tests of liver function are variably abnormal including increased serum bilirubin and increased hepatic enzymes. Tests for carbohydrate and lipid metabolites are frequently abnormal depending on the degree of liver disease.

☑ CHECKPOINT 15-13

What are the causes of macrocytosis seen in alcoholism?

Stimulated Erythropoiesis

Increased erythropoietin (stimulation) in the presence of an adequate iron supply (e.g., autoimmune hemolytic anemia) can result in the release of shift reticulocytes from the bone marrow. These cells are larger than normal with an MCV as high as 130 fL. A reticulocyte count and examination of the blood smear allow distinction of this macrocytic entity from megaloblastic anemia. In the presence of large numbers of shift reticulocytes, polychromasia is markedly increased. The WBC count is slightly increased, and the platelet count is normal. In addition, the oval macrocytes typical of megaloblastic anemia are not present in conditions associated with increased erythropoietin stimulation.

Hypothyroidism

Anemia of hypothyroidism presents as a mild to moderate anemia with a normal reticulocyte count. Thyroid hormone regulates cellular metabolic rate and therefore tissue oxygen requirement. With a decrease in thyroid hormone (i.e., hypothyroidism), tissue oxygen requirement is reduced and the kidneys adapt appropriately. The net result is a decrease in the production of EPO and, correspondingly, erythrocytes. Thus although the hemoglobin concentration is reduced, the tissue oxygenation is adequate. This type of "anemia" often presents as a macrocytic, normochromic anemia but can also be a normocytic, normochromic anemia. The anemia can be complicated by iron, folic acid, or B_{12} deficiency, and the blood picture can reflect these forms of anemia.

☑ CHECKPOINT 15-14

What are three clinical or laboratory findings (in addition to assessing the bone marrow) that can distinguish a non-megaloblastic macrocytic anemia from a megaloblastic anemia?

Summary

Macrocytosis due to megaloblastic anemia must be differentiated from macrocytosis with a normoblastic marrow. The laboratory test profile of a patient with megaloblastic anemia commonly indicates pancytopenia. The blood smear reveals macro-ovalocytes (i.e., large ovalocytes), poikilocytosis, teardrop cells, Howell-Jolly bodies, and neutrophil hypersegmentation. The marrow is characterized by megaloblastosis of precursor cells due to a block in thymidine production. Because thymidine is one of the four DNA bases, the deficiency leads to a diminished capacity for DNA synthesis and a block in mitosis. The marrow is hypercellular, but erythropoiesis is ineffective. Causes of megaloblastosis are nearly always due to cobalamin or folic acid deficiencies.

Folate is primarily acquired from the diet. The liver is the main storage site of folic acid. Folate deficiency results in decreased synthesis of N^5, N^{10}-methylene THF, a cofactor in DNA synthesis. Consequently, DNA synthesis slows. Clinically, symptoms from inadequate dietary folate can occur within months as compared with years after onset of a cobalamin deficiency.

Vitamin B_{12} (i.e., cyanocobalamin) absorption occurs in the small intestine. Dietary cobalamin is released from digestion of animal proteins in meats and bound by gastric haptocorrins and subsequently intrinsic factor (IF). Once absorbed, cobalamin is bound to specific plasma proteins known as *transcobalamin* and *haptocorrins*. Normal serum cobalamin values are highly variable based on age and sex. Cobalamin function is related to DNA synthesis because cobalamin is a vital cofactor in the conversion of methyl tetrahydrofolate to tetrahydrofolate. This product is an important cofactor needed for the production of DNA thymidine.

Defective production of intrinsic factor is the most common cause of cobalamin deficiency (pernicious anemia [PA]), which is caused by failure of the gastric mucosa to secrete IF. PA most commonly occurs in people after 40 years of age. Central nervous system symptoms can be present in advanced cases. Laboratory tests used to diagnose PA include serum cobalamin, MMA, IFBA, serum gastrin, and the Schilling test. Other causes of cobalamin deficiency include gastrectomy, malabsorption diseases such as Crohn's disease, and drugs. Megaloblastic anemia rarely results from chemotherapeutic drugs or congenital enzyme deficiencies or with other hematopoietic diseases such as congenital dyserythropoietic anemia.

Serum and RBC folate can be measured to diagnose folate deficiency, but the tests do not reliably indicate folate stores in the presence of cobalamin deficiency. Cobalamin levels can be directly assessed by measuring serum cobalamin or indirectly by measuring MMA in the urine/serum/plasma or homocysteine in plasma.

Macrocytosis due to megaloblastic anemia must be differentiated from macrocytosis with a normoblastic marrow. Normoblastic, macrocytic anemias can result from acute blood loss or hemolysis due to shift reticulocytosis from the marrow. Alcohol abuse is one of the most common causes of normoblastic macrocytosis. Liver disease, often resulting from alcohol abuse, is also commonly associated with macrocytosis without a megaloblastic marrow.

Review Questions

Level I

1. The most common cause of macrocytosis is: (Objective 5)
   A. folate deficiency
   B. alcoholism
   C. liver disease
   D. pernicious anemia
2. In the majority of cases, cobalamin deficiency is due to a deficiency of: (Objective 6)
   A. intrinsic factor
   B. vitamin B_6
   C. folate
   D. methylmalonic acid
3. Which of the following is the best clue in diagnosing megaloblastic anemia? (Objective 3)
   A. decreased hemoglobin and hematocrit
   B. leukocytosis
   C. hypersegmented neutrophils
   D. poikilocytosis
4. Increases in urinary excretion of formiminoglutamic acid (FIGLU) most likely indicate which of the following? (Objective 4)
   A. cobalamin deficiency
   B. autoantibodies to intrinsic factor
   C. folic acid deficiency
   D. hemolysis
5. The liver stores enough folate to meet daily requirement needs for how long? (Objective 2)
   A. 1 month
   B. 6–8 weeks
   C. 2 years
   D. 3–6 months
6. Which of the following conditions increases the daily requirement for cobalamin? (Objective 2)
   A. pregnancy
   B. aplastic anemia
   C. hypothyroidism
   D. splenectomy
7. A deficiency of cobalamin leads to impaired: (Objective 1)
   A. folic acid synthesis
   B. DNA synthesis
   C. intrinsic factor secretion
   D. absorption of folate
8. Laboratory diagnosis of pernicious anemia can include which of the following? (Objective 3)
   A. urinary FIGLU
   B. WBC count
   C. gastric analysis
   D. LDH
9. Alcoholic individuals commonly develop a macrocytic anemia due to: (Objective 8)
   A. folate deficiency
   B. increased blood cholesterol levels
   C. development of autoantibodies against intrinsic factor
   D. intestinal malabsorption of cobalamin
10. Anemia due to liver disease is often associated with which of the following RBC morphological forms? (Objective 9)
    A. ovalocytes
    B. microcytes
    C. spur cells
    D. teardrop cells

Level II

1. If a patient presents with anemia, macrocytosis, pancytopenia, and malnutrition, which of the following should be investigated first as a possible cause of the anemia? (Objectives 4, 12)
   A. pernicious anemia
   B. folic acid deficiency
   C. cobalamin deficiency
   D. celiac disease
2. Which laboratory tests are most sensitive to decreased levels of cobalamin? (Objectives 8, 11)
   A. red cell and serum folate
   B. serum cobalamin and red cell folate
   C. transcobalamin assays
   D. MMA and homocysteine

3. Which of the following can be found in a patient with megaloblastic anemia? (Objectives 2, 8, 11)
   A. giant metamyelocytes and hypolobulated neutrophils
   B. Howell-Jolly bodies and Pappenheimer bodies
   C. hypersegmented neutrophils and oval macrocytes
   D. hypochromic macrocytes and thrombocytosis
4. The metabolic function of tetrahydrofolate is to: (Objective 1)
   A. synthesize methionine
   B. transfer carbon units from donors to receptors
   C. serve as a cofactor with cobalamin in the synthesis of thymidylate
   D. synthesize intrinsic factor
5. Folic acid deficiency can be caused by: (Objective 6)
   A. alcoholism
   B. chronic blood loss
   C. strict vegetarian diet
   D. vitamin B_6 deficiency
6. A lack of intrinsic factor could be due to: (Objective 7)
   A. gastrectomy
   B. cobalamin deficiency
   C. folate deficiency
   D. large bowel resection
7. Which of the following is more typical of nonmegaloblastic than megaloblastic anemia? (Objectives 2, 3)
   A. oval macrocytes
   B. round macrocytes
   C. Howell-Jolly bodies
   D. hypersegmented neutrophil
8. If a patient excretes <7.5% of a radioactively tagged crystalline B_{12} dose after 24 hours both before and after administration of a dose of oral intrinsic factor, which of the following is a possible diagnosis? (Objective 9)
   A. pernicious anemia
   B. liver disease
   C. gastrectomy
   D. celiac disease
9. Which type of congenital dyserythropoietic anemia (CDA) presents with giant multinucleated erythrocytes in the marrow? (Objective 10)
   A. CDA I
   B. CDA II
   C. CDA III
   D. CDA IV
10. A 48-year-old Caucasian female experiencing fatigue, loss of appetite, and weight loss over a period of three months was seen by her physician. A medical history revealed she had a history of alcohol abuse. An initial laboratory workup demonstrated that she was anemic, had a leukocyte count of 3×10^9/L, and had an MCV of 119 fL. Macro-ovalocytes and neutrophil hypersegmentation were noted on her blood smear evaluation. Based on the initial laboratory test results, her physician obtained a serum cobalamin and folate workup. Results were as follows:

Serum cobalamin	550 pg/mL
Serum folate	4.0 ng/mL
RBC folate	105 ng/mL

Based on her clinical history and laboratory results, the best possible diagnosis is which of the following? (Objective 12)

   A. pernicious anemia
   B. folate deficiency
   C. primary cobalamin deficiency
   D. anemia of liver disease

Companion Resources

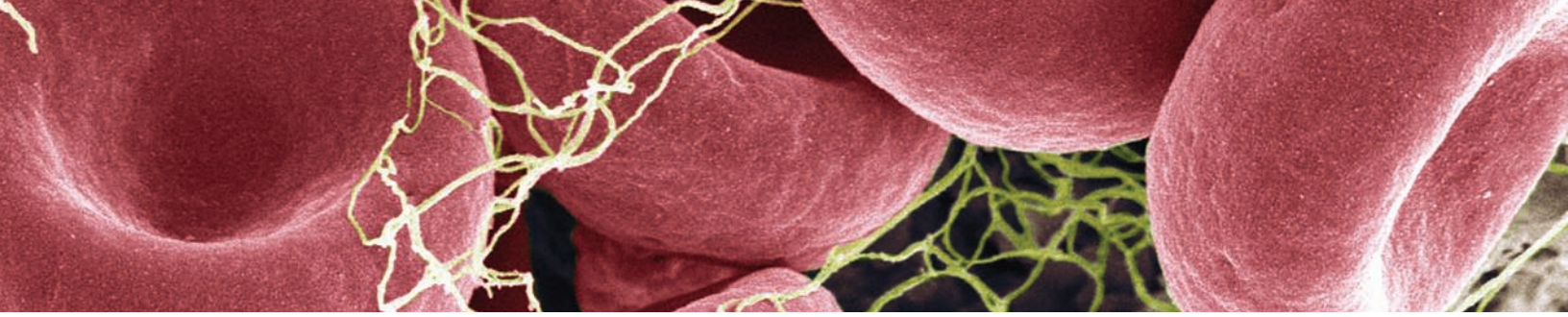
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

The views expressed in this chapter are those of the author and do not necessarily reflect the official policy or position of the Department of the Army, the Department of Defense, or the U.S. Government.

References

1. Hoggarth K. Macrocytic anaemias. *Practitioner*. 1993;237(1525):331–32,335.
2. Colon-Otero G, Menke D, Hook CC. A practical approach to the differential diagnosis and evaluation of the adult patient with macrocytic anemia. *Med Clin North Am*. 1992;76:581–97.
3. Kass L. Pernicious anemia. In: Smith LH Jr, ed. *Major Problems in Internal Medicine*. Vol. 7. Philadelphia: W.B. Saunders; 1976:1–62.
4. Addison T. *On the Constitutional and Local Effects of Disease of the Suprarenal Capsules*. London, England: Sam Highley; 1855:43.
5. Ehrlich P. *Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes*. Berlin, Germany: A. Hirschwald; 1891:137.
6. Hvas A, Nexo E. Diagnosis and treatment of vitamin B_{12} deficiency. An update. *Haematologica*. 2006;91:1506–12.
7. Carmel R. Macrocytosis, mild anemia, and delay in the diagnosis of pernicious anemia. *Arch Intern Med*. 1979;139:47–50.
8. Hall CA. Vitamin B_{12} deficiency and early rise in mean corpuscular volume. *JAMA*. 1981;245:1144–46.
9. Spivak JL. Masked megaloblastic anemia. *Arch Intern Med*. 1982;142: 2111–14.
10. Carmel R, Weiner JM, Johnson CS. Iron deficiency occurs frequently in patients with pernicious anemia. *JAMA*. 1987;257:1081–83.
11. Chan CWJ, Liu SYH, Kho CJB et al. Diagnostic clues to megaloblastic anemia without macrocytosis. *Intern J Lab Hematol*. 2007;29(3):163–71.
12. Pruthi RK, Tafferi A. Pernicious anemia revisited. *Mayo Clin Proc*. 1994;69:144–50.
13. Lindenbaum J, Healton EB, Savage DG et al. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *N Engl J Med*. 1988;318:1720–28.
14. Green R. Folate, cobalamin, and megaloblastic anemias. In: Kaushansky K, Lichtman MA, Beutler E et al., eds. In *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010; 533–63.
15. Klee GG. Cobalamin and folate evaluation: Measurement of methylmalonic acid and homocysteine vs vitamin B_{12} and folate. *Clin Chem*. 2000;46(8 Pt 2):1277–83.
16. Schrier SL. Diagnosis and treatment of vitamin B_{12} and folate deficiency. UpToDate©. www.uptodate.com; accessed July 22, 2013.
17. Antony AC Megaloblastic anemias. In: R Hoffman, EJ Benz Jr, LE Silberstein et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Saunders; 2013:491–524. Chapter 37.
18. Jacques PF, Selhub J, Bostom AG et al. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med*. 1999;340:1449–54.
19. MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet*. 1991;338(8760):131–37.
20. Chanarin I. Megaloblastic anaemia, cobalamin, and folate. *J Clin Pathol*. 1987;40:978–84.
21. Elin RJ, Winter WE. Methylmalonic acid: a test whose time has come. *Arc Path Lab Med*. 2001;125(6):824–27.
22. Hsing AW, Hansson L-E, McLaughlin JK, et al. Pernicious anemia and subsequent cancer: a population-based cohort study. *Cancer*. 1993;71:745–50.
23. Carson-Dewitt RS. Pernicious anemia. In *Gale Encyclopedia of Medicine*. Farmington Hills, MI: The Thomson Corp.; 2002;2224.
24. Oh R, Brown OL. Vitamin B_{12} deficiency. *Am Fam Phys*. 2003;67(5):979–92.
25. Legan RC, Zawistoski KJ. Update on vitamin B_{12} deficiency. *Am Fam Physician*. 2011;83(12):1425–30.
26. Carmel R, Melnyk S, James SJ. Cobalamin deficiency with and without neurologic abnormalities: differences in homocysteine and methionine metabolism. *Blood*. 2003;101(8)3302–8.
27. Welch GN, Loscalzo J. Homocysteine and atherothrombosis. *N Engl J Med*. 1998;338:1042–50.
28. Ridker PM, Hennekens CH, Selhub J et al. Interrelation of hyperhomocyst(e)inemia, factor V Leiden, and risk of future venous thromboembolism. *Circulation*. 1997;95:1777–82.
29. Holleland G, Schneede J, Ueland PM et al. Cobalamin deficiency in general practice: assessment of the diagnostic utility and cost-benefit analysis of methylmalonic acid determination in relation to current diagnostic strategies. *Clin Chem*. 1999;45:189–98.
30. Mayo Clinic. Pernicious anemia cascade, serum. www.mayomedicallaboratories.com. 03 Mar. 2005. Web; accessed July 21, 2014.
31. Valente E, Scott JM, Ueland PM et al. Diagnostic accuracy of holotranscobalamin, methylmalonic acid, serum cobalamin, and other indicators of tissue vitamin B_{12} status in the elderly. *Clin Chem*. 2011;57(6):856–63.
32. Carmel R. Biomarkers of cobalamin (vitamin B_{12}) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *Am J Clin Nutr*. 2011; 94(1):348S–58S.
33. Nexo E, Hoffmann-Lucke E. Holotranscobalamin, a marker of vitamin B_{12} status: analytical aspects and clinical utility. *Am J Clin Nutr*. 2011;94(1):359S–65S.
34. Kumar V. Pernicious anemia. *MLO*. 2007;39(2):28–30.
35. Norman EJ, Morrison JA. Screening elderly populations for cobalamin (vitamin B_{12}) deficiency using the urinary methylmalonic acid assay by gas chromatography mass spectrometry. *Am J Med*. 1993;94:589–94.
36. Havas AM, Morkbak AL, Nexo E. Plasma holotranscobalamin compared with plasma cobalamins for assessment of vitamin B_{12} absorption; optimization of a non-radioactive vitamin B_{12} absorption test (CobSorb). *Clin Chim Acta*. 2007;376(1–2):150–54.
37. Balaman Z, Kadikoylu G, Yukselen V et al. Oral versus intramuscular cobalamin treatment in megaloblastic anemia: a single-center, prospective, randomized open-label study. *Clin Ther*. 2003;25(12):3124-34.
38. Wilson A, Leclerc D, Rosenblatt DS et al. Molecular basis for methionine synthase reductase deficiency in patients belonging to the cblE complementation group of disorders in folate/cobalamin metabolism. *Hu Mol Gene*. 1999;8:2009–16.
39. Seppa K, Sillanaukee P, Saarni M. Blood count and hematologic morphology in nonanemic macrocytosis: differences between alcohol abuse and pernicious anemia. *Alcohol*. 1993;10:343–47.
40. Katz R, Cooper GW, Gordon AS, Zanjani ED. Studies on the site of production of erythropoietin. *Ann NY Acad Sci*. 1968;149:120–27.



16 Hypoproliferative Anemias

REBECCA LAUDICINA, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *hypoproliferative anemia*.
2. Cite the diagnostic criteria for aplastic anemia.
3. Describe the epidemiology and etiology of aplastic anemia.
4. Explain the pathophysiology of aplastic anemia.
5. Compare and contrast acquired and inherited aplastic anemia.
6. List the major clinical and laboratory characteristics of aplastic anemia.
7. Identify environmental factors associated with the development of aplastic anemia.
8. Describe the etiology, bone marrow, and peripheral blood in pure red cell aplasia.
9. Identify peripheral blood findings associated with the following: aplastic anemia, pure red cell aplasia, and anemia due to renal disease.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Propose and explain possible causes of aplastic anemia.
2. Discuss prognosis in aplastic anemia.
3. Compare methods of treatment and management of patients with aplastic anemia.
4. Contrast aplastic anemia with other causes of pancytopenia on the basis of clinical findings and peripheral blood and bone marrow findings.
5. Compare and contrast pure red cell aplasia with aplastic anemia and other causes of erythroid hypoproliferation.
6. Compare and contrast major characteristics of Diamond-Blackfan syndrome and transient erythroblastopenia of childhood.
7. Explain the pathophysiology of anemia due to renal disease.
8. Evaluate laboratory test results and medical history of a clinical case for a patient with hypoproliferative anemia, suggest additional laboratory tests if appropriate, and a possible diagnosis.

Chapter Outline

Key Terms

Aplasia
Aplastic anemia
Diamond-Blackfan anemia (DBA)
Dyshematopoiesis
Fanconi anemia (FA)
Hypocellularity
Hypoplasia
Hypoproliferative
Idiopathic
Myelophthisis
Pancytopenia
Pure red cell aplasia

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe basic laboratory procedures used to screen for and assess anemia. (Chapters 10, 11)
- Identify abnormal values and results for basic hematologic laboratory procedures. (Chapter 10)
- Outline the classification of anemias. (Chapter 11)
- Explain the process of hematopoiesis. (Chapter 4)

Level II

- Explain the concepts of stem cell renewal and differentiation. (Chapter 4)
- Describe the normal bone marrow structure and cellularity and the process of bone marrow examination. (Chapters 3, 38)

CASE STUDY

We will address this case study throughout the chapter.

Rachael, a 13-year-old female, was admitted to the hospital with complaints of progressive weakness and shortness of breath with minimal physical effort. She has experienced recurrent fevers reaching 102°F. Physical examination revealed a well-developed adolescent with good nutritional status and in no acute distress. There was no lymphadenopathy or organomegaly. Many petechial hemorrhages covered her chest and legs. Several bruises were found on her legs and thighs. Laboratory tests were ordered upon admission.

Consider the diagnostic possibilities in this case and how laboratory tests can be used to assist in differential diagnosis.

OVERVIEW

Finding cytopenias in the peripheral blood often suggests the presence of a serious medical condition. Although there are a variety of causes, bone marrow hypoproliferation is one of the most serious. This chapter discusses the acquired and inherited anemias that are associated with bone marrow hypoproliferation. The first section describes the classification of hypoproliferative anemias followed by discussions of aplastic anemia and pure red cell aplasia. These anemias are contrasted to other causes of cytopenias.

INTRODUCTION

The **hypoproliferative** anemias are a heterogeneous group of acquired and inherited disorders in which there is a normocytic or macrocytic, normochromic anemia associated with chronic bone marrow **hypocellularity**. Much of the area in the bone marrow normally occupied by hematopoietic tissue is replaced by fat. The terms *aplastic*, **aplasia**, and *hypoplastic* refer to a bone marrow with an overall decrease in hematopoietic cellularity. If there is **hypoplasia** of only one of the hematopoietic lineages, the terms *erythroid*, *myeloid*, or *megakaryocytic hypoplasia* should be used to define the specific entity.

The hematopoietic defect is due to depletion, damage, or inhibition of hematopoietic stem cells (HSCs) and/or hematopoietic progenitor cells (HPCs). Either the unipotent erythroid progenitor cell or a multilineage hematopoietic progenitor cell can be affected. The peripheral blood findings provide important clues to help identify the bone marrow abnormality. If only the erythroid progenitor cells (BFU-E, CFU-E) are affected, platelets and leukocytes remain normal, and the diagnosis is **pure red cell aplasia**. More commonly, the multilineage hematopoietic precursor cells (HSC, MPP, CMP, CFU-GEMM, etc.) are affected, resulting in **pancytopenia** (decreases of all three cell lineages), and the diagnosis is **aplastic anemia**.

APLASTIC ANEMIA

The term *aplastic anemia* is used to describe the condition of pancytopenia that is associated with a hypocellular bone marrow. The mature blood cells that are produced in aplastic anemia (AA) usually appear normal. Aplasia of the bone marrow is only one of several possible causes of peripheral blood pancytopenia, but pancytopenia due to causes other than AA can result in morphologically abnormal blood or bone marrow cells. Whereas AA is usually characterized by pancytopenia, granulocyte, platelet, and erythrocyte, levels may not be depressed uniformly.

Diagnostic criteria for severe aplastic anemia are included in Table 16-1 ★. Additional diagnostic criteria are based on disease severity.[1] With disease progression, concentrations of cells in all three cell lineages eventually become further depleted. This reflects an impaired proliferative capacity of the marrow stem cells, which lose their ability for normal cellular renewal.

★ **TABLE 16-1** Diagnostic Criteria for Aplastic Anemia

Bone marrow cellularity <25%, plus two of the following:
Granulocyte count $<0.5 \times 10^9$/L
Platelet count $<20 \times 10^9$/L
Anemia with corrected reticulocyte count <1% (absolute concentration $<40 \times 10^9$/L)

CASE STUDY *(continued from page 303)*

1. Select laboratory tests appropriate for screening for aplastic anemia.
2. Justify the selection of laboratory screening tests based on Rachael's clinical signs and symptoms.

☑ CHECKPOINT 16-1

An anemic patient has a (corrected) reticulocyte count of 1.5%, hemoglobin of 100 g/L, hematocrit of 0.30 L/L, total neutrophil count of 0.4×10^9/L, and a platelet count of 30×10^9/L. Is it likely that this patient has aplastic anemia?

Epidemiology

The anemias described in this chapter are rare, occurring at a frequency of two cases per million. The actual prevalence of aplastic anemia may be somewhat higher due to underdiagnosis and imprecision in applying diagnostic standards.[1] A geographic variation in incidence occurs with 2–3 times more cases in Asia than in western countries. It is believed that this variation is related to environmental and occupational factors.[2] Individual susceptibility could also play a role. Aplastic anemia is most commonly seen in 15–25-year-olds with two smaller affected groups consisting of 2–5 year olds and adults older than 60. The occurrence of AA in young children is mostly due to inherited forms, whereas the occurrence in adults is most frequently due to acquired forms, although there are exceptions.[1,3]

Pathophysiology

Recent attention has been focused on immunologic suppression of hematopoiesis as a cause of acquired aplastic anemia.[4] Response of marrow failure to immunosuppressive therapy has led to the characterization of most cases of acquired AA as an immune-mediated disease.[5] The immune mechanism responsible is thought to involve the suppression of HSC growth and differentiation by abnormal cytotoxic T lymphocytes. This is supported by finding that treatment of the majority of AA patients with immunosuppressive drugs, including antithymocyte globulin (ATG) and cyclosporine results in clinical improvement.[6]

Immunosuppressive therapy presumably eliminates an abnormal population of activated cytotoxic T lymphocytes that produces interferon-γ (IFN-γ) and tumor necrosis factor (TNF), substances known to inhibit hematopoiesis.[4] One mechanism of inhibition is induction of apoptosis, resulting in cell death. Both IFN-γ and TNF induce overexpression of Fas (a cell membrane receptor) by HSCs and HPCs. When the Fas receptor is activated by binding with its ligand, apoptosis is initiated (Chapter 2). It is estimated that the number of HSCs in severe acquired AA is decreased to <1% of normal. In tissue culture, the addition of lymphocytes taken from patients with AA decreases hematopoietic cell production, and their removal improves the production of hematopoietic cells, thereby supporting the immunosuppression theory.[7]

Flow cytometric analysis of lymphocyte subpopulations in acquired aplastic anemia has revealed a marked increase in activated $CD8^+$ lymphocytes. The cause of activation of T lymphocytes in AA remains unclear, although it may be associated with viral infections or medical drugs in small numbers of cases. There is also evidence supporting a genetic basis of aberrant cytotoxic T lymphocyte activation. An increased incidence of AA is associated with the presence of the class II histocompatibility antigen DR2, which is also linked to other autoimmune disorders. Aplastic anemia patients positive for this antigen respond well to immunosuppressive therapy.[8]

The success of hematopoietic stem cell transplants (SCT) in many patients with AA indicates that the pancytopenia can be corrected by repopulation of the marrow with normal HSCs and immune cells. The aberrant clone of T lymphocytes is eliminated through the pretransplant conditioning regimen and subsequently replaced with normal cells, thus lending further support to the immune basis of aplastic anemia.[4]

Defective telomere maintenance in HSCs and HPCs has been observed in some patients with AA and may contribute to its pathophysiology. Telomeres are the ends of chromosomes consisting of 500–2000 tandem repeats of the hexanucleotide TTAGGG. The telomere ends of chromosomes shorten with each cell division (Chapter 2). Maintenance of telomere length occurs through the action of the enzyme telomerase. Mutations in telomerase-related genes, including telomerase reverse transcriptase (TERT), telomerase RNA template (TERC), and shelterin (TINF) result in shortened telomeres, reduced proliferation of hematopoietic precursors, and increased apoptosis. Furthermore, shortened telomeres may predispose to malignant transformation, which is observed in some patients with AA. Additional environmental or genetic factors can also contribute to telomere shortening. Shortened telomeres and the mutations associated with them have been observed in patients with both acquired and inherited forms of aplastic anemia.[9]

Classification and Etiology

Aplastic anemia can be classified as either acquired or inherited (Table 16-2 ★). Historically, much attention has focused on an association between acquired AA and environmental exposures. Drugs, chemicals, radiation, infectious agents, and other factors have been linked to the development of acquired AA, which can be temporary or persistent. In most cases, no environmental link can be identified, and the cause is said to be **idiopathic**. The immune pathophysiologic model (discussed in the previous section) provides a unifying basis for understanding the disorder regardless of the presence or absence of environmental factors. Although acquired AA is more common

★ **TABLE 16-2** Classification of Hypoproliferative Anemias

Aplastic Anemia	Pure Red Cell Aplasia	Other Hypoproliferative Anemias
Acquired	Transitory infections	Anemia of chronic renal disease
Idiopathic	Acquired pure red cell aplasia	Anemia associated with endocrine abnormalities
Drugs: chloramphenicol, phenylbutazone, gold compounds, sulfa drugs, antihistamines, antithyroid, tetracyclines, penicillin, methylphenylethylhydantoin	Acute: infections, transient erythroblastopenia of childhood (TEC), drugs	
Chemical agents: benzene, insecticides, hair dye, carbon tetrachloride, chemotherapeutics (vincristine, busulfan, etc.), arsenic	Chronic: thymoma, autoimmune disorders	
Ionizing radiation		
Biological agents: parovirus, infectious mononucleosis, infectious hepatitis, measles, influenza, errors of amino acid metabolism, starvation		
Pregnancy		
Paroxysmal nocturnal hemoglobinuria		
Inherited	**Inherited**	
Fanconi anemia	Diamond-Blackfan anemia	
Familial aplastic anemia		
Dyskeratosis congenital		
Congenital amegakarocytic thrombocytopenia		
Shwachman-Diamond syndrome		

in adults, it is also an infrequent cause of aplasia in children. Both idiopathic and other acquired forms of AA are discussed in the next sections.

Inherited forms of AA result in chronic failure of the bone marrow, which may or may not be present at birth. A congenital condition is one that is manifest early in life, often at birth, but that is not necessarily inherited; it can be caused by acquired factors (e.g., maternal exposure to an environmental toxin while pregnant). Inherited AA is quite rare and is often associated with other congenital anomalies.

CASE STUDY *(continued from page 304)*

3. Evaluate the relationship between Rachael's age and the likelihood that she has aplastic anemia.
4. If aplastic anemia is present, would you expect her to have an idiopathic or secondary form? Explain your answer.

Acquired Forms of Aplastic Anemia

Idiopathic

The majority of cases of aplastic anemia cannot be linked to an environmental factor and are referred to as *idiopathic*. It is possible that previous exposure to an unrecognized agent or event could be responsible for stimulating the immune system.

Drugs and Chemical Agents

Recent research has shown that exposure to drugs or chemical agents is rarely associated with aplastic anemia, although historically these associations were given prominent attention. A study conducted in Thailand, where the incidence of AA is 2–3 times higher than in the United States, indicated that an elevated risk of developing AA was associated with exposure to only a small number of substances, including sulfonamides, thiazide diuretics, and mebendazole. In the Thai study, an increased risk was not found for chloramphenicol, a drug frequently implicated in case reports of AA.[10] Other drugs that have been implicated include gold, anticonvulsants, nonsteroidal analgesics, antiprotozoals, and antithyroid medications.[1] Most individuals taking such medications, however, do not develop AA. One possible explanation is that persons with diminished P-glycoprotein, an efflux pump that is the product of the multi-drug resistance gene *MDR-1*, may have excessive accumulation of drugs that can increase susceptibility to HSC damage.[11] In cases associated with drug exposure, the pathophysiology is thought to involve an abnormal immune response to the HSC.

The widespread use of toxic chemical agents in industry and agriculture is probably responsible for some cases of bone marrow aplasia. Benzene derivatives are well established as a cause of bone marrow suppression. Although most cases develop within a few weeks after exposure, some occur after months or years of chronic exposure. Although stem cells can be damaged, the main toxic effect of benzene is usually expressed on transient stages of committed proliferating precursor cells. Elevated risk of AA was documented in the Thai study for persons exposed to benzene and other solvents as well as agricultural pesticides such as organophosphates and DDT and for persons who drink nonbottled water in rural regions.[10]

Most of the cytotoxic drugs used in chemotherapy of malignant diseases kill rapidly proliferating cells. However, the drugs do not distinguish between malignant and normal cells. Therefore, all proliferating cells are damaged, including normal cells of the hematopoietic compartment. Although quiescent (G_0) stem cells are spared from immediate drug exposure, repeated doses of the drug over a long period of time can eventually deplete the remaining HSCs as they enter the proliferating pool. In most cases, timely withdrawal of the drug leads to hematopoietic recovery.

Ionizing Radiation

Aplastic anemia has been encountered in persons exposed to ionizing radiation in industrial accidents, military nuclear tests, and therapeutic regimens for malignancy. Ionizing radiation is directly toxic to HSCs and HPCs, and the effects are dose dependent. Small doses affect all cells but are especially destructive to rapidly proliferating cells. The bone marrow can recover from sublethal doses of irradiation because quiescent stem cells are induced to begin proliferating after exposure-induced depletion of more differentiated progeny. Therapy in these cases is mostly supportive and needed only until hematopoietic function is restored. With high doses (>4000 rads), bone marrow aplasia and peripheral blood pancytopenia are usually permanent.

Infectious Agents

Viral and bacterial infections can be followed by a transient cytopenia. The aplasia can be limited to the erythroid elements or can include all three cell lineages. Aplasia has been described in patients after recovery from infectious mononucleosis, tuberculosis, and hepatitis but is typically transient. Hepatitis-associated AA occurs most frequently in males and does not appear to be caused by any of the known hepatitis viruses. It is often fatal if untreated.[12] An aplastic crisis in patients with hereditary hemolytic anemias is commonly associated with human parvovirus infection. This aplasia, however, is limited to the erythroid lineage.

In Epstein-Barr viral infections as well as other viral infections, the virus can infect the HSC, triggering an immune response. Cytotoxic lymphocytes then destroy the virus-infected stem cell. Other mechanisms of stem cell damage in viral infections have been postulated, including direct cytotoxicity of the virus and inhibition of cellular proliferation and differentiation.[4]

Metabolic

The rare inborn errors of amino acid metabolism, which result in accumulation of ketones and glycine, have been associated with aplastic anemia.

Starvation or protein deficiency results in hypoproliferative anemia after about 3 months of deprivation. Starvation that is not self-induced usually occurs in areas where other endemic pathologies are also present, such as parasitic infection and blood loss. Thus, the causes of this anemia can be multifactorial.

Decreased hormonal stimulation of hematopoietic precursor cells is important primarily as a factor in erythroid hypoplasia. Renal disease and endocrine diseases are examples of hypoproliferation caused by a decrease in erythropoietin.

A life-threatening pancytopenia rarely can occur during pregnancy. The condition usually remits after delivery or abortion. It has been suggested that the aplasia may be related to estrogen inhibition of stem cell proliferation.[13]

Association with Clonal Disorders

Even prior to the widespread use of immunosuppressive therapy for AA, patients occasionally developed clonal disorders, including *paroxysmal nocturnal hemoglobinuria (PNH)* and myelodysplastic syndromes (MDS). Thus, the development of these disorders in AA patients is not necessarily related to the treatment regimens currently used. As many as 15% of patients treated with immunosuppressive therapy for aplastic anemia may develop MDS.[1]

PNH is an acquired stem cell disease in which a blood cell membrane abnormality increases the cells' susceptibility to in vivo complement-mediated hemolysis. PNH is caused by mutations in the *PIGA* gene resulting in the absence of a glycolipid that serves to attach and anchor proteins to the cell membrane. Although considerable variation in clinical manifestations occurs, the typical picture in severe cases is pancytopenia and marrow hypoplasia. Between 40–50% of patients with an initial diagnosis of AA develop an abnormal erythrocyte population similar to that seen in PNH, but they do not usually develop the full clinical manifestations of PNH (intravascular hemolysis and venous thrombosis).[14] PNH is a prominent, late complication in many AA patients who have received immunosuppressive ATG therapy.[15] PNH is discussed in detail in Chapter 20.

CASE STUDY *(continued from page 305)*

For the past 3 months, Rachael's family physician has been following her recovery from viral hepatitis. Her recovery was uneventful; her liver enzyme levels returned to normal within 2 months. She has no other past medical history. There is no family history of hematologic disorders.

5. What aspect of this patient's history could be associated with the occurrence of aplastic anemia?

Inherited Aplastic Anemia

A number of disorders have a congenital predisposition for developing AA, as described below.

Fanconi Anemia

Fanconi anemia (FA) is an autosomal recessive (or rarely X-linked recessive) disorder resulting from a variety of molecular defects and is characterized by childhood-onset aplastic anemia, abnormal chromosomal fragility, and an increased predisposition to developing leukemia and cancer. FA has a prevalence of about 1–5 per million persons in North America with a frequency of the heterozygous carrier state of ~1 in 300.[16] Patients can have a complex assortment of congenital anomalies in addition to progressive bone marrow hypoplasia. The congenital defects include dysplasia of bones, renal abnormalities, and other organ malformations as well as mental retardation, dwarfism, microcephaly, hypogonadism, and skin hyperpigmentation. Some patients lack congenital defects and may not be diagnosed until AA develops, which occasionally may not occur until adulthood.[17]

Aplastic anemia eventually develops in ~90% of FA patients. The hematological manifestations are generally slowly progressive from birth and need to be monitored closely. Clinical signs of pancytopenia usually appear between the ages of 5 and 10 years with the median age at diagnosis of 6.5 years.[3] Anemia is usually macrocytic (although it can be normocytic) with macrocytosis often preceding anemia. Erythrocytes often show increased levels of Hemoglobin F and the i antigen (reflecting hematologic stress and the development of erythrocytes from earlier erythroid progenitor cells). Leukopenia primarily

involves the granulocytes. Thrombocytopenia often precedes anemia and leukopenia. Androgen therapy can reverse the pancytopenia for several years in about 50% of FA patients. G-CSF therapy can help increase neutrophil counts.

HSCT offers the only possible curative treatment for bone marrow failure in FA patients. In patients with HLA-matched sibling donors, 5-year survival is close to 75%. Stem cells from HLA-matched unrelated donors have also been used. Because FA patients are acutely sensitive to chemotherapeutic agents and radiation, pretransplant conditioning protocols must be altered to reduce toxicity that could increase the risk of developing a clonal disorder. Although HSCT can cure the marrow failure in FA patients, the increased risk of nonhematologic malignancies remains.

The risk for developing cancer in FA is high and increases with age. Acute nonlymphocytic leukemia and solid tumors are common complications. Leukemia is difficult to treat, and survival is poor. With improved treatment options, the median survival for FA patients is now 29 years of age.[3]

Karyotyping of FA cells shows increased spontaneous chromosomal breakage, gaps, rearrangements, exchanges, and duplications. Diagnosis of FA involves exposing blood lymphocytes or skin fibroblasts to DNA crosslinking agents such as mitomycin C, diepoxybutane (DEB), or cisplatin, which amplify chromosomal breakage.[1,3] Molecular techniques can be used to identify specific mutations. Testing for FA is performed when warranted based on clinical findings and/or abnormal blood counts, usually in infants and children.

The molecular defects in FA are heterogeneous. To date, mutations in 13 genes that are associated with the FA phenotype have been identified. Several of the FA-identified proteins function in a common cellular DNA repair mechanism, which is activated in response to DNA damage. Defective FA proteins are thus responsible for the genetic instability associated with DNA damage, resulting in increased apoptosis of hematopoietic stem cells and AA.[1,3] In addition to genetic defects leading to defective DNA repair and DNA instability, TNFα is elevated in most patients. The telomeres of the HSC and HPC are shortened, suggesting an abnormally high proliferative rate, probably induced by the marrow's attempt to replace damaged HSCs/HPCs and ultimately lead to HSC/HPC premature senescence.[9]

Other Causes of Inherited Aplastic Anemia

Several other rare disorders have a predisposition to developing AA. *Dyskeratosis congenita (DC)* is an inherited bone marrow failure syndrome, resulting in AA in ~50% of cases. The physical abnormalities and mutations in patients with DC differ from those seen in patients with FA. Most DC patients have dystrophic nails and leukoplakia of the oral mucosa, conditions not seen in FA. Mutations in several genes are associated with DC. Markedly reduced telomerase activity, telomere shortening, early HSC/HPC senescence, and a reduced stem cell compartment are characteristic. There are multiple subtypes of DC with varying clinical features.[3]

Congenital amegakaryocytic thrombocytopenia (CAT) presents in the neonatal period with isolated thrombocytopenia due to reduced or absent marrow megakaryocytes. Aplastic anemia subsequently develops in ~45% of patients, usually in the first years of life. The defect is a mutation in the *MPL* gene, which codes for the thrombopoietin (TPO) receptor.[3] TPO is required for maintenance of HSC viability (Chapter 4).

Patients with *Shwachman-Diamond Syndrome (SDS)* can present with some of the same congenital anomalies, including AA as is seen in FA patients. However, they also have exocrine pancreatic dysfunction and malabsorption syndrome and do not display increased chromosomal fragility and defective DNA repair processes. This disorder is due to mutations of the *SBDS* gene, although the exact pathogenesis of the disorder is still unknown. Diagnosis is confirmed by genetic testing.[3]

CASE STUDY *(continued from page 306)*

6. Is it likely that Rachael has an inherited form of aplastic anemia? Explain your answer.

Clinical Findings

The onset of symptoms in AA is usually insidious and related to the cytopenias. Common initial signs are bleeding accompanied by petechial and mucosal hemorrhages and infection. Pallor, fatigue, and cardiopulmonary complications can be present as the anemia progresses.

Hepatosplenomegaly and lymphadenopathy are absent. Splenomegaly has occasionally been noted in later stages of the disease, but if found in the early stages, the diagnosis of aplastic anemia should be questioned.

Laboratory Findings

Laboratory studies of peripheral blood and bone marrow are essential if a diagnosis of aplastic anemia is suspected.

Peripheral Blood

Pancytopenia is typical. Although the degree of severity can vary, the diagnosis of AA should be questioned unless the leukocyte count, erythrocyte count, and platelet count are all below the reference intervals. Hemoglobin is usually <70 g/L. Erythrocytes appear normocytic and normochromic, or they can be slightly macrocytic. The presence of nucleated erythrocytes and teardrops is not typical of AA but suggests marrow replacement (myelophthisic anemia). Myelodysplastic syndrome, rather than AA, is suggested by the presence of dysplastic neutrophils and other abnormal cells. The relative reticulocyte count (%) can be misleading due to the severe anemia. Therefore, the reticulocyte count should always be determined in absolute concentration and/or be corrected for anemia before interpretation. The absolute reticulocyte count is usually $<25 \times 10^9$/L. The corrected reticulocyte count is $<1\%$. Most often, thrombocytopenia is present at the time of diagnosis. Neutropenia precedes leukopenia; initially, lymphocyte and monocyte counts are normal. Because of the neutropenia, the differential count reflects a relative lymphocytosis. When the leukocyte count is below 1.5×10^9/L, an absolute lymphocytopenia is also present. The band to segmented neutrophil ratio is increased, and occasionally more immature forms are found. Neutrophil granules are frequently larger than normal and stain a dark red; these granules should be distinguished from toxic granules, which are bluish black. Flow cytometry of the peripheral blood can be ordered to detect CD59+ cells when PNH is suspected[18] (Chapter 20).

CASE STUDY *(continued from page 307)*

7. Correlate Rachael's clinical findings of weakness and shortness of breath as well as petechial hemorrhages and bruises with her laboratory screening results, which follow.

   Admission laboratory data for patient:

RBC	2.42×10^{12}/L
Hb	71 g/L
Hct	0.24 L/L
PLT	8.0×10^{9}/L
WBC	1.2×10^{9}/L
Differential	
Segmented neutrophils	2%
Lymphocytes	94%
Monocytes	4%
Uncorrected reticulocyte count	0.7%

8. Evaluate each of Rachael's laboratory results by comparing them to reference intervals.
9. Which of Rachael's routine laboratory results are consistent with those expected for aplastic anemia?
10. Classify the morphologic type of anemia.
11. Calculate the absolute lymphocyte count. Are Rachael's lymphocytes truly elevated as suggested by the relative lymphocyte count?
12. Correct the reticulocyte count. Why is this step important?
13. Calculate the absolute reticulocyte count.

Bone Marrow

Examination of the bone marrow is necessary to differentiate aplastic anemia from other diseases accompanied by pancytopenia. In AA, the bone marrow is hypocellular with >70% fat (Figure 16-1 ■). Thus, it is often difficult to obtain an adequate sample. Bone marrow infiltration with granulomas or cancer cells can lead to fibrosis, also resulting in a hypocellular dry tap on aspiration. Both aspiration and biopsy are needed for a correct diagnosis.[18] It is recommended that several different sites be aspirated because focal sampling of the marrow can be misleading. Some areas of acellular stroma and fat can be infiltrated with clusters of lymphocytes, plasma cells, and reticulum cells. Areas of residual hematopoietic tissue termed *hot spots* can be found primarily early in the disease but can occasionally be found in severe refractory cases. Iron staining reveals many iron granules in macrophages, but granules are rarely seen in normoblasts. Flow cytometry should be performed; the percentage of CD34+ cells in the bone marrow in AA is typically <0.3%. Bone marrow karyotyping is useful for differentiating hypocellular forms of myelodysplastic syndromes from aplastic anemia.[1]

CASE STUDY

Rachael was referred to a hematologist who ordered a bone marrow examination. The aspirate obtained was inadequate for evaluation due to lack of marrow spicules. Only a single site was aspirated. Touch preps made from the biopsy showed a markedly hypocellular marrow with very few hematopoietic cells. Cells present consisted of lymphocytes, plasma cells, and stromal cells. There were no malignant cells present.

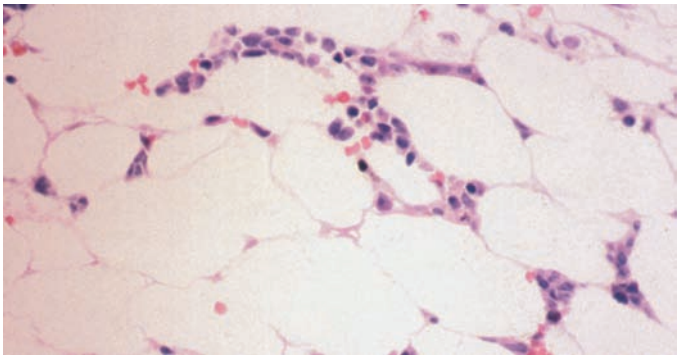
14. Compare these results with those expected for a person with aplastic anemia.
15. Interpret the significance of the lack of malignant cells and hematopoietic blasts.
16. Suggest a way to improve the validity of bone marrow examination results for this patient.

Other Laboratory Findings

Other abnormal findings are not specific for aplastic anemia but are frequently found associated with the disease. Hemoglobin F can be increased, especially in children. Erythropoietin is often increased, particularly when compared with the erythropoietin levels in patients with similar degrees of anemia. Serum iron is increased with >50% saturation of transferrin, reflecting erythroid hypoplasia. The clearance rate of iron (Fe^{59}) from the plasma is decreased because of the decrease in iron utilization by a hypoactive marrow. Patients who are younger than age 50 should be screened for FA using tests for chromosomal breakage.[18] Results of these tests will be normal in other forms of inherited AA and in acquired/idiopathic forms of AA.

Prognosis and Therapy

Recent advances in treatment have tempered the previously grim prognosis of patients diagnosed with aplastic anemia. HSCT and immunosuppressive therapy (IST) have greatly improved survival. Presently,



■ **FIGURE 16-1** Bone marrow preparation from a patient with aplastic anemia shows marked hypocellularity (10%). The patches of cells remaining are primarily lymphocytes (Wright-Giemsa stain; 100× magnification).

the 5-year survival rate is 79%.[19] Choice of definitive therapy for severe acquired AA depends on the age of the patient and availability of a matched donor. HSCT is recommended for patients up to age 45 who have a matched sibling donor, although some recommendations extend the age limit to age 55. HSCT is also recommended for patients up to age 21 with a fully compatible HLA-matched unrelated donor. IST is recommended for patients without a matched related donor, the situation faced by the majority of patients with aplastic anemia.[20]

Before beginning HSCT or IST, putative causative drugs should be withdrawn or the patient removed from a hazardous environment. The immediate treatment is often supportive with the administration of erythrocytes, platelets, and antibiotics. Granulocyte transfusions can be given to severely neutropenic patients with life-threatening sepsis.[18] To avoid alloimmunization, transfusion should be minimized if HSCT is anticipated. Irradiated and/or leukocyte-reduced blood products can be ordered (Chapter 1). Hematopoietic growth factors such as G-CSF generally do not have a beneficial effect on patients with AA and may increase the risk of MDS.[5]

HSCT using cells collected from bone marrow has become a relatively common procedure and is curative in many patients with aplastic anemia. Bone marrow is preferred over peripheral blood stem cells due to the increased risk of graft-vs-host disease with the latter. Antithymocyte globulin (ATG) and cyclosporine are typically used as preconditioning regimens to suppress the immune response of the recipient. Preconditioning is nonmyeloablative.[20] The current 5-year survival rate is 77% for HLA-matched sibling donors. Survival rates are highest for children and for patients who have been minimally transfused.[4] Treatment complications and post-transplant mortality remain high, especially in older patients; therefore, the probability for long-term cure must be weighed against the inherent risks of complications, including graft-vs-host disease and early and late toxicities of the conditioning regimen. Although engraftment of HSCs is successful in many cases, some transplants, even when performed between identical twins, do not correct the AA. These unsuccessful transplants suggest that the donor HSC growth is suppressed by the same immune mechanism that induced the original aplasia (Chapter 29). An additional constraint to HSCT is that matched sibling donors are available for only 20–30% of patients with aplastic anemia. Combined, intensive immunosuppressive therapy (IST) using ATG in combination with cyclosporine has become standard treatment for those patients with acquired severe aplastic anemia who lack a suitable bone marrow donor. IST is effective in restoring hematopoiesis in 60–90% of patients who are over 45 or who lack an HLA-matched sibling donor.[4,18] More favorable outcomes are observed in children. Relapse requiring additional IST occurs in 30–40% of patients. About 15% of patients treated with IST develop clonal disorders such as PNH, leukemia, and myelodysplastic syndromes.[4]

CASE STUDY *(continued from page 308)*

17. Appraise the prognosis for Rachael.
18. Predict a treatment regimen.

Differentiation of Aplastic Anemia from other Causes of Pancytopenia

Pancytopenia can be associated with disorders other than AA, but these disorders differ in that the pancytopenia is not the result of a defect in stem cell proliferation. Rather, the bone marrow can be normocellular, hypercellular, or infiltrated with abnormal cellular elements.

Myelophthisic Anemia

Myelophthisis signifies marrow replacement or infiltration by fibrotic, granulomatous, or neoplastic cells (Chapter 21). The abnormal replacement cells reduce normal hematopoiesis and disrupt the normal bone marrow architecture, allowing the release of immature cells into the peripheral blood. Anemia can be accompanied by normal, increased, or decreased leukocyte and platelet counts. The most characteristic findings are a leukoerythroblastic reaction and a moderate to marked poikilocytosis. Dacryocytes are common, as are large bizarre platelets. By contrast, nucleated erythrocytes and significant morphologic changes are almost never found in the peripheral blood in AA. Myelophthisic anemia is associated with diffuse cancer of the prostate, breast, and stomach and is typical of myelofibrosis and lipid storage disorders.

Myelodysplastic Syndromes

Myelodysplastic syndromes (MDS) are a group of hematological disorders that have a propensity to terminate in acute leukemia (Chapter 25). The principal peripheral blood findings are pancytopenia, bicytopenia, or isolated cytopenias with reticulocytopenia. A macrocytic anemia is common. The bone marrow, however, is usually normocellular or hypercellular with various degrees of qualitative abnormalities of one or more cell lineages (**dyshematopoiesis**). Signs of dyshematopoiesis are also reflected in morphologic abnormalities of one or more cell lineages in the peripheral blood. These qualitative abnormalities of peripheral blood cells are useful in differentiating cytopenias due to true hypoproliferation of stem cells (AA) from cytopenias due to ineffective erythropoiesis.

A subgroup of patients with MDS can have a hypocellular marrow. This condition is referred to as *hypoplastic MDS* (*HMDS*). In these cases, differentiation of MDS and AA can be complicated if there are few bone marrow cells from a biopsy or aspirate to examine. If granulocytic and megakaryocytic dysplasias are seen and/or if an abnormal karyotype consistent with MDS is present, HMDS can be diagnosed. However, if only erythroid dysplasia is present, the evidence is insufficient to differentiate HMDS and AA because erythroid dysplasia can be found in some cases of AA.[15]

Congenital Dyserythropoietic Anemia

Congenital dyserythropoietic anemia (CDA) is a very rare familial refractory anemia characterized by both abnormal and ineffective erythropoiesis. The bone marrow is normocellular or hypercellular, but the peripheral blood is pancytopenic. In contrast to the normal erythroid precursors found in aplastic anemia, erythrocyte precursors in the bone marrow of CDA patients exhibit multinuclearity, and myeloblasts and promyelocytes are increased. The anemia can be normocytic, but most often it is macrocytic. The three types of CDA are discussed in Chapter 15.

Hypersplenism

Hypersplenism due to a variety of causes can result in a decrease of one or more cellular elements of the blood as these elements become pooled and sequestered in the spleen. In this condition, the bone marrow is hyperplastic, corresponding to the peripheral blood cytopenia. Anemia is accompanied by a reticulocytosis as opposed to the reticulocytopenia found in the true hypoproliferative anemias. Granulocytopenia can be accompanied by a shift to the left in these cells. Splenomegaly and other findings of the underlying disease are important in diagnosing this disorder. Splenectomy, although not always advisable, corrects the cytopenias.

Other

Deficiency of cobalamin or folic acid can be accompanied by pancytopenia (Chapter 15). The bone marrow in these cases, however, reveals normocellularity or hypercellularity with megaloblastic changes. In the peripheral blood, hypersegmented neutrophils and Howell-Jolly bodies are typical but are not found in pancytopenia of aplastic anemia.

☑ CHECKPOINT 16-2

A pancytopenic patient has a presumptive diagnosis of aplastic anemia. While performing a smear review, you observe the presence of several types of poikilocytes, dysmorphic neutrophils, and large agranular platelets. Do these findings support the presumptive diagnosis? If not, which disorder(s) would be more likely for this patient?

CASE STUDY (continued from page 309)

19. What other hematologic conditions must be ruled out for Rachael?
20. What laboratory test is most beneficial in differentiating aplastic anemia from these other disorders? Compare the expected results of AA with those of the other disorders.

PURE RED CELL APLASIA

Pure red cell aplasia (PRCA) is a heterogeneous group of disorders characterized by a selective decrease in erythroid precursor cells in the marrow and by peripheral blood anemia. The anemia can be severe and is typically normocytic, normochromic. PRCA is thought to occur due to selective hypoproliferation of the committed erythroid progenitor cell. The term *aplastic anemia* should be avoided in describing PRCA because there is no disturbance of granulopoiesis or thrombopoiesis. Reticulocytes can be present but are <1% when corrected for the degree of anemia. PRCA can be acquired (acute or chronic) or inherited and can affect any age group. Acquired PRCA can develop in patients with thymoma, hematopoietic neoplasms, other cancers, autoimmune disorders, infection following administration of certain drugs, immunosuppressive therapy, and post-transplantation.[21] Transient erythroblastopenia of childhood (TEC) is an acquired, self-limiting form of erythroid hypoplasia found in children. Inherited forms of PRCA, including Diamond-Blackfan anemia, are discussed in the section "Diamond-Blackfan Anemia." The underlying pathophysiology of PRCA is variable and depends on associated clinical conditions. Treatment of the underlying condition can resolve the anemia, but unresponsive cases are considered to have an immune basis.[22] Refer to Table 16-3 ★ for results of laboratory tests in PRCA.

★ **TABLE 16-3** Laboratory Findings in Pure Red Cell Aplasia

Test	Findings
Erythrocyte count, hematocrit, and hemoglobin	Decreased
Reticulocyte count	Severely decreased
Leukocyte count	Normal or possibly increased
Platelet count	Normal or possibly increased
Bilirubin	Low or normal
Bone marrow	Absence of erythroid cells if examined early in disease; if examined later in disease progression, increase in young erythroid cells, which can be mistaken for an erythroid maturation arrest. If the patient is followed, however, these cells show normal maturation and differentiation. There is normal myelopoiesis and granulopoiesis.

Acquired Acute Pure Red Cell Aplasia

Viral (parvovirus, Epstein-Barr virus, and viral hepatitis) and/or bacterial infections can be associated with a temporary suppression of erythropoiesis. B19 parvovirus infects erythroid progenitor cells, lyses the target cell, and blocks erythropoiesis. Infection, as well as the block to erythropoiesis, is terminated by the production of neutralizing antibodies to the virus. In individuals who have a normal erythrocyte life span and hemoglobin level, temporary erythroid hypoproliferation is not noticed. However, if the erythrocyte life span is decreased due to an underlying condition, the complication of sudden erythroid hypoproliferation (aplastic crisis) can be life threatening. These aplastic crises are most frequently noted in patients with hemolytic anemias including sickle cell anemia, paroxysmal nocturnal hemoglobinuria, and autoimmune hemolytic anemias.

The aplastic crisis cases brought to the attention of a physician probably represent only a minor fraction of the actual occurrence of temporary erythroid aplasia. The aplastic crises are often preceded by fever with upper respiratory and intestinal complaints. Several members of the same family are frequently affected with the illness. Patients with concurrent hemolytic anemia have a rapid onset of lethargy and pallor. Patients without hemolytic anemia may seek medical attention because of the primary illness, and anemia is only an incidental finding.

If the anemia is severe, supportive therapy of packed erythrocyte transfusions may be necessary until spontaneous recovery occurs. PRCA associated with viral hepatitis has a poor prognosis.

Acute erythroid hypoplasia also occurs with the administration of some drugs and chemicals. After removal of the inciting agent, normal erythropoiesis usually resumes.

Transient erythroblastopenia of childhood (TEC) is a form of acquired acute pure red cell aplasia, but because of the importance of distinguishing this pediatric anemia from Diamond-Blackfan anemia (DBA), TEC will be discussed with DBA in the section "Diamond-Blackfan Anemia."

Chronic Acquired Pure Red Cell Aplasia

An acquired selective depression of erythroid precursors is a rare disorder encountered in middle-aged adults. This disease usually occurs in association with thymoma, autoimmune hemolytic anemia, systemic lupus erythematosus, rheumatoid arthritis, or hematologic neoplasms (MDS, large granular lymphocytic leukemia, chronic lymphoid leukemia, Hodgkin disease). The high incidence of autoimmune disorders associated with this disorder suggests that an immunologic mechanism could be responsible for the red cell aplasia. Cytotoxic antibodies to erythropoietin-sensitive cells in the marrow and to erythropoietin have been demonstrated in some cases. More commonly, the mechanism appears to be a T cell-mediated immunosuppression of erythropoiesis. In some cases, PRCA may be the initial presenting sign of the underlying disorder.[21]

Clinical findings are nonspecific. Pallor is usually the only physical finding. Therapies include transfusion with packed erythrocytes, thymectomy if the thymus is enlarged, and immunosuppression. Various drugs, including corticosteroids, cyclosporine, cytotoxic agents, ATG, and monoclonal antibodies against B lymphocytes (rituximab/anti-CD20, alemtuzumab/anti-CD52), have been used to suppress the immune response. Androgens or erythropoietin to stimulate erythrocyte production is rarely used. In drug-induced red cell aplasia, withdrawal of the putative agent is indicated. Approximately 80% of patients with acquired PRCA have a spontaneous remission or remission induced by immunosuppression. About half will relapse, but with additional immunosuppression, 80% will enter a second remission. By retreating relapsing patients or continuing maintenance immunosuppression, many can be maintained transfusion-free for years.[22] HSCT is rarely indicated for acquired pure red cell aplasia.

Diamond-Blackfan Syndrome

Diamond-Blackfan anemia (DBA) is a rare congenital progressive erythrocyte aplasia that occurs in very young children. Anemia is present at birth in 25% of cases, and in 98% of cases, diagnosis is made within the first year of life.[21] Unlike Fanconi anemia, there is no leukopenia or thrombocytopenia. The anemia is typically severe.

DBA is actually a diverse family of diseases with a common hematologic phenotype. Evidence exists for both autosomal dominant and autosomal recessive modes of inheritance. About 20–25% of cases have a mutation of the *RPS19* gene (which encodes for a protein involved in ribosome assembly). Other genes mutated in patients with DBA have been identified and although the basic defect in most cases appears to be defective ribosome biogenesis, the exact functional defect in all mutations is not clear. A wide range of physical abnormalities can be observed in approximately 50% of DBA patients, and patients are predisposed to occurrences of cancer.[21] Many cases have no familial pattern, suggesting spontaneous mutations or acquired disease.

The anemia in DBA is not due to a deficiency of erythropoietin (EPO) because EPO levels are consistently increased and actually higher than expected for the degree of anemia. The EPO is active, and no antibodies directed against this cytokine are found, nor are there apparent mutations in either EPO or the EPO receptor. The probable defect in DBA is an intrinsic defect of erythroid progenitor cells, resulting in their inability to respond normally to inducers of proliferation and differentiation. Hence, there is typically a deficit of erythroid precursors in the marrow of patients with DBA.[21] Physical findings and symptoms are those associated with anemia or related to the congenital defects. Anemia is severe with erythroid hypoplasia in an otherwise normocellular bone marrow.

Diagnostic criteria for DBA are included in Table 16-4 ★.[21] Testing for *RPS19* mutations is helpful in only about 20–25% of cases. Testing for the chromosomal breakage associated with FA is negative. Vitamin B_{12} and folate levels are normal. Fetal-like erythrocytes are present; hemoglobin F is increased to 5–25%, and the i antigen is increased. The presence of fetal-like erythrocytes is not particularly useful in diagnosing DBA in children younger than 1 year of age because children in this age group normally possess erythrocytes with fetal characteristics. Serum iron and serum ferritin are increased, and transferrin is 100% saturated.

Therapy includes erythrocyte transfusions and administration of adrenal corticosteroids. Up to half of DBA patients develop prolonged remission, but most eventually require additional therapy. Transfusion dependence requires iron-chelation therapy (deferroximine). Most deaths are due to complications of therapy such as hemosiderosis.

DBA must be distinguished from transient erythroblastopenia of childhood (TEC), a temporary suppression of erythropoiesis that frequently occurs after a viral infection in otherwise normal children (Table 16-5 ★). The age of onset of TEC ranges from 1 month to 10 years with most cases occurring in children over the age of 2. Progressive pallor in a previously healthy child

★ **TABLE 16-4** Diagnostic Criteria for Diamond-Blackfan Anemia

Macrocytic (or normocytic) normochromic anemia in first year of life
Reticulocytopenia
Normocellular marrow with marked erythroid hypoplasia
Increased serum erythropoietin (EPO)
Normal or slightly decreased leukocyte count
Normal or increased platelet count

★ **TABLE 16-5** Comparison of the Features of Diamond-Blackfan Anemia and Transient Erythroblastopenia of Childhood (TEC)

Feature	Diamond-Blackfan	TEC
PRCA	Present	Present
Fetal erythrocyte characteristics (i antigen, HbF increased)	Present	Absent (if child is >1 year old)
Etiology	Inherited	Acquired following viral infection (mechanism unknown)
MCV	Can be increased	Normal
Age at onset	Birth to 1 year	>1 year
Physical abnormalities	Present in 50%	Absent or unrelated to anemia
Therapy	Corticosteroids, transfusions	None, spontaneous recovery, or 1–2 transfusions

is the primary clinical finding. Fetal characteristics of erythrocytes (HbF, i antigen) seen in DBA are not found in TEC. The pathophysiology of TEC is thought to be either a virus-associated, antibody-mediated, or a T cell-mediated suppression/inhibition of erythroid precursors. Although a preceding viral infection is associated with TEC, parvovirus B19 is not the etiologic agent. It is important that the distinction be made between DBA and TEC because DBA requires treatment that is unnecessary and potentially harmful to children with TEC. Patients with TEC recover within 2 months of diagnosis. Therapy, if needed, usually involves 1– 2 red cell transfusions[21]

OTHER HYPOPROLIFERATIVE ANEMIAS

Other hypoproliferative anemias due primarily to defective hormonal stimulation of erythroid progenitor cells include the anemia associated with chronic renal disease and the anemias associated with endocrine disorders. In most cases, these anemias can be traced to a decrease in erythropoietin production.

Renal Disease

Chronic renal disease is a common cause of anemia. The patient usually seeks medical attention for symptoms related to renal failure, and the anemia is discovered during the initial workup. The hemoglobin begins to decrease when the blood urea nitrogen level increases to >30 mg/dL. Anemia develops slowly, and most patients tolerate the low hemoglobin levels well.

Pathophysiology

Due to the complexity of the clinical settings in uremia, anemia is frequently the result of several different pathophysiologies (Table 16-6 ★):

1. The most important and consistent factor is bone marrow erythroid hypoproliferation attributed to a decrease in erythropoietin (EPO) production by the diseased kidney.
2. In some cases, the EPO level is normal, but the bone marrow does not respond. The unresponsiveness can be caused by the presence of a low molecular weight dialyzable inhibitor of erythropoiesis present in the serum of uremic patients. In these cases, improvement in hemoglobin levels is seen after dialysis.
3. In addition to hypoproliferation, decreased erythrocyte survival compounds the anemia. One factor responsible for the shortened survival is related to an unknown extracorpuscular cause, perhaps an unfavorable metabolic environment or mechanical trauma. Another cause of hemolysis can be related to an acquired abnormality in erythrocyte metabolism that involves the pentose phosphate shunt. This abnormality causes impaired generation of NADPH and reduced glutathione.[23] Thus, when exposed to oxidants, the erythrocytes develop Heinz bodies, inducing acute hemolysis. Hemolysis can also be related to a reversible defect in erythrocyte membrane sodium-potassium ATPase.
4. The anemia can be related to blood loss from the gastrointestinal tract because of a decrease in platelets and/or platelet dysfunction. Blood is also lost during priming for dialysis. Patients receiving dialysis lose about 5–6 mg of iron daily. Thus, an anemia complicated by iron deficiency is common (Chapter 12).
5. Patients on dialysis can become folate deficient because folate is dialyzable. Without folate supplements, the patient can develop a megaloblastic anemia (Chapter 15).

Laboratory Findings

A normocytic and normochromic anemia is typical in renal disease except when the patient is deficient in folate or iron; then a macrocytic anemia or microcytic anemia prevails. Moderate anisocytosis with some degree of microcytosis can be present. Hemoglobin levels

★ **TABLE 16-6** Possible Causes of Anemia in Chronic Renal Disease

Decreased erythropoietin production
Presence of an inhibitor of erythropoiesis
Decreased erythrocyte survival
Blood loss
Iron deficiency
Folate deficiency

are reduced to 50–80 g/L, and the reticulocyte production index is approximately 1. There is moderate to severe poikilocytosis with echinocytes and schistocytes. The number of echinocytes correlates roughly with the severity of azotemia. Spherocytes are associated with hypersplenism. Nucleated erythrocytes are noted in the peripheral blood. Leukocytes and platelets are usually normal. The bone marrow reveals erythroid hypoproliferation, especially when compared with the degree of anemia.[23]

Other laboratory findings vary depending on the severity of renal impairment. Typically blood urea nitrogen is >30 mg/dL, serum creatinine is increased, and electrolytes are abnormal. Hemostatic abnormalities can be present. Serum ferritin levels are higher than normal in chronic renal failure even if iron deficiency is present. Therefore, it has been suggested that if the serum ferritin level is <40 ng/mL, iron deficiency should be considered. Increased iron-binding capacity can be a useful predictor of iron deficiency in these cases.

Therapy

Therapy for chronic renal disease includes hemodialysis, continuous ambulatory peritoneal dialysis, and renal transplantation. All treatments tend to ameliorate the anemia, but hemodialysis exposes the patient to additional causes of anemia including blood loss, iron and folate deficiency, and hemolysis. Thus, iron and folic acid supplements are frequently given in conjunction with hemodialysis. Intermittent doses of EPO three times a week cause improvement in 1–2 weeks. In some cases, a normal hemoglobin is achieved, and in all cases, the patients remain transfusion-independent.

Endocrine Abnormalities

Disorders of the thyroid, adrenals, parathyroid, pituitary, or gonads can result in a mild to moderate anemia.[24] Endocrine deficiencies are sometimes associated with a decrease in EPO. The resulting anemia is usually normocytic, normochromic with normal erythrocyte morphology. The bone marrow findings suggest erythroid hypoproliferation. Treatment of the endocrine disorder can resolve the anemia.

A slowly developing normocytic, normochromic anemia is characteristic of hypothyroidism. Erythrocyte survival is normal, and reticulocytosis is absent. The anemia is most likely a physiologic response to a decrease in tissue demands for oxygen. With hormone replacement therapy, the anemia slowly remits.

Hypopituitarism is associated with an anemia more severe than that of hypothyroidism, and the leukocyte count can be decreased. However, anemia is a minor component of the other manifestations of hypopituitarism. The pituitary has an effect on multiple endocrine glands including the thyroid and adrenals. In males, a decrease in androgens (gonadal dysfunction) can be partly responsible for the anemia because they stimulate erythropoiesis. In addition, a decrease in growth hormone can have a trophic effect on the bone marrow. Mild anemia has also been associated with hyperparathyroidism.

Summary

The hypoproliferative anemias include a group of acquired and inherited disorders in which a chronic marrow failure of erythropoiesis occurs. If only the erythrocytes are affected, the term *pure red cell aplasia* is appropriate. More commonly, hypocellularity involves all cell lineages, and the diagnosis is aplastic anemia.

Immune suppression has been shown to frequently underlie the hypocellularity in acquired aplastic anemia. Acquired AA can be idiopathic or secondary to drugs, chemical agents, ionizing radiation, or infectious agents. Inherited AA can be associated with other congenital anomalies. Fanconi anemia is a form of inherited aplastic anemia with progressive bone marrow hypoplasia and other congenital defects. The disorder is characterized by chromosomal instability and fragility, secondary to defective DNA repair mechanisms.

The laboratory findings in AA reveal pancytopenia. The erythrocytes are usually normocytic and normochromic but can be macrocytic. The reticulocyte count is low, and the corrected reticulocyte count is <1%. The bone marrow is <25% cellular.

Pure red cell aplasia is characterized by a selective decrease in erythroid cells. This disorder can be acquired or inherited. The acquired forms are seen in thymoma, with administration of certain drugs, autoimmune disorders, and infection, especially viral infections. DBA is an inherited progressive erythrocyte aplasia occurring in young children. This inherited form of aplasia must be differentiated from TEC, a temporary aplasia occurring after viral infection in otherwise healthy children.

Other hypoproliferative anemias are due primarily to defective hormonal stimulation of erythroid stem cells. These include anemia associated with renal disease and with endocrinopathies. The laboratory findings reflect not only anemia but also pathologies of the primary disorder.

Immunosuppressive therapy using antithymocyte globulin, cyclosporine, or monoclonal antibodies against B-cell antigens is the treatment of choice for the majority of older patients who are not candidates for hematopoietic stem cell transplants (HSCT). HSCT is potentially curative but not without risks. Transplantation still remains unavailable for many patients due to the inability to find matched donors.

Review Questions

Level I

1. Which statement best explains current theory on the etiology of aplastic anemia? (Objective 3)

   A. An immune mechanism, possibly involving abnormal T lymphocytes, leads to suppression of hematopoietic stem cells.

   B. A deficiency of cytokines, such as erythropoietin, results in hematopoietic stem cells which are incapable of mitosis.

   C. Hematopoietic stem cells are crowded out by neoplastic cells invading the bone marrow.

   D. The bone marrow microenvironment is damaged and rendered nonsupportive of the growth of hematopoietic stem cells.

2. A 60-year-old patient with a history of severe anemia was found to have a marked decrease in erythrocyte precursors in the bone marrow with no dysplasia. Leukocyte and platelet precursors were normal. Her hemoglobin was 74 g/L, reticulocyte count was decreased, and red cell indices were normal. Erythrocyte morphology was normal. These test results and patient presentation are consistent with which disorder? (Objectives 8, 9)

   A. aplastic anemia

   B. iron-deficiency anemia

   C. sickle cell disease

   D. pure red cell aplasia

3. A 6-year-old child has CBC and bone marrow results consistent with a diagnosis of aplastic anemia. Chromosome fragility was present, and genetic mutations were demonstrated by molecular analysis. Which disorder is most consistent with this scenario? (Objectives 5, 6)

   A. Congenital dyserythropoietic anemia

   B. Diamond-Blackfan anemia

   C. Fanconi anemia

   D. Acquired aplastic anemia

4. Which laboratory test result is *inconsistent* with a diagnosis of aplastic anemia? (Objective 9)

   A. bone marrow cellularity = 50%

   B. granulocyte count = 0.2×10^9/L

   C. platelet count = 15×10^9/L

   D. reticulocyte count = 0.3%

5. Which statement best describes current research on potential exposure to environmental agents and the development of aplastic anemia? (Objective 7)

   A. There is *no* documented association between any environmental factors and development of aplastic anemia.

   B. The majority of aplastic anemia cases are *not* linked to environmental exposures, but links to benzene and radiation exposure and some medications have been reported.

   C. Environmental factors associated with development of most cases of aplastic anemia include a wide array of infectious and chemical agents, drugs, and radiation.

   D. Only the antibiotic chloramphenicol and radiation exposure are tied to aplastic anemia.

6. What is the typical morphologic classification of erythrocytes in aplastic anemia? (Objective 9)

   A. hypochromic, microcytic

   B. normochromic, normocytic

   C. normochromic, microcytic

   D. hypochromic, macrocytic

7. The bone marrow in aplastic anemia is typically: (Objective 8)

   A. hypocellular

   B. hypercellular

   C. dysplastic

   D. normal

8. Which of the following is (are) considered a cause of hypoproliferation in aplastic anemia? (Objective 3)

   1. damage to stem cells
   2. depletion of stem cells
   3. inhibition of stem cells

   A. 1 only

   B. 1 and 2 only

   C. 2 and 3 only

   D. 1, 2, and 3

9. What term best describes the peripheral blood findings of a person with aplastic anemia? (Objective 9)

   A. pancytopenia

   B. bicytopenia

   C. granulocytopenia only

   D. anemia only

10. Diagnostic criteria for aplastic anemia include: (Objective 2)
    A. corrected reticulocyte count of $>1\%$
    B. platelet count $<100 \times 10^9$/L
    C. granulocyte count $<0.5 \times 10^9$/L
    D. bone marrow $<50\%$ cellular

Level II

1. What next line of testing would you recommend be ordered when a previously healthy adult presents with pancytopenia on a routine automated CBC? (Objective 8)
    A. molecular assays for FA mutations
    B. chromosome breakage assay
    C. review of RBC morphology on the peripheral blood smear
    D. bone marrow examination
2. Immunosuppressive therapy (IST) can be used when patient has a diagnosis of aplastic anemia. What is the relationship between this treatment and a prevalent theory on pathophysiology of the disorder? (Objectives 1, 3)
    A. Current theory holds that the cause of AA is due to hematopoietic stem cells suppression by abnormal cytotoxic T lymphocytes, which are sensitive to IST.
    B. Current theory is that AA is due to neoplastic changes in hematopoietic stem cells, which must be eliminated by IST.
    C. Because current theory emphasizes the role of environmental factors such as toxin and drug exposure, IST enhances immune function and helps the individual with AA mount an immune response.
    D. There is no evidence of a relationship between the cause of AA and IST; we know only empirically that it is effective.
3. A 70-year-old patient with a recent history of severe anemia, thrombocytopenia, and neutropenia was referred to a hematologist. Further testing revealed a marked decrease in all hematopoietic precursors in the bone marrow with no dysplasia. Pancytopenia and severely low hemoglobin was observed in the peripheral blood. Red cell indices and erythrocyte morphology was normal. These test results and patient presentation are consistent with which disorder? (Objective 8)
    A. aplastic anemia
    B. iron-deficiency anemia
    C. anemia of chronic disease
    D. pure red cell aplasia
4. Mr. Garcia is a 68-year-old man who is being evaluated for kidney disease. His blood urea nitrogen (BUN) is 15 mmol/L (reference interval = 2.1–7.1 mmol/L). His hemoglobin concentration was found to be 109 g/L. Which statement is the best possible explanation for his hemoglobin concentration? (Objectives 5,7,8)
    A. He most likely has experienced severe blood loss (hemorrhage) as result of kidney disease.
    B. His kidney disease is causing decreases in erythropoietin, red cell survival, iron, and folate, resulting in anemia.
    C. Mr. Garcia's hemoglobin concentration is normal for a male his age and is unrelated to kidney disease.
    D. Nothing in the clinical or laboratory data presented explains the hemoglobin and additional testing is required.
5. What are drawbacks to treatment of aplastic anemia with hematopoietic stem cell transplants? (Objective 3)
    A. This is considered merely supportive therapy and must be continuously administered.
    B. Not all patients will respond to this therapy, which is most successful with older patients.
    C. Treatment complications and post-transplant mortality are high.
    D. This treatment is recommended only for very young children and is not useful for adults.
6. What confirmatory test should be performed in suspected cases of AA? (Objective 4)
    A. serum iron and TIBC
    B. hemoglobin electrophoresis
    C. bone marrow examination
    D. direct antiglobulin test
7. A 3-year-old patient presents with severe normocytic, normochromic anemia. Platelet counts and leukocyte counts are normal. The mother reported that the child has been healthy since birth but recently had a cold. Which of the following laboratory test results would support a diagosis of TEC? (Objectives 1, 5, 6)
    A. decreased numbers of erythrocyte precursors on bone marrow examination
    B. normal hemoglobin F level on hemoglobin electrophoresis
    C. abnormal karyotype
    D. i antigen on the patient's erythrocytes

8. A bone marrow from an anemic patient that demonstrates a marked erythroid hypoplasia but normal numbers of other cell lineages is most consistent with a diagnosis of: (Objective 5)

   A. Fanconi anemia

   B. aplastic anemia

   C. pure red cell aplasia

   D. myelophthisic anemia

9. A male patient with previously diagnosed infectious mononucleosis infection has become suddenly anemic. A possible cause of the anemia is: (Objective 1)

   A. iron deficiency

   B. folic acid deficiency

   C. anemia of chronic disease

   D. aplastic anemia

10. What is the standard treatment for patients with acquired aplastic anemia? (Objective 3)

    A. immunosuppressive therapy

    B. bone marrow transplant

    C. administration of growth factors

    D. none; treatment typically not needed

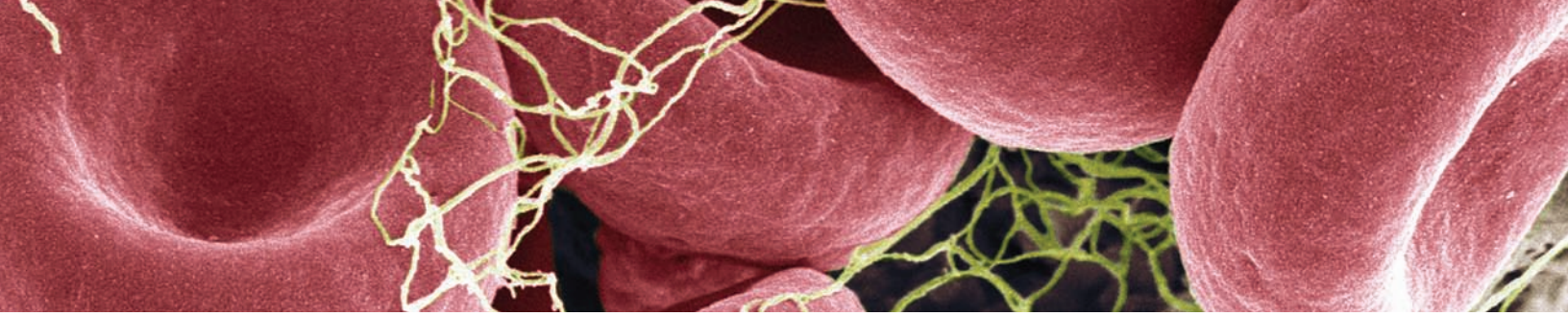
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Brodsky RA. Acquired aplastic anemia. In: Greer JP, Foester J, Rodgers GM et al., eds. *Wintrobe's Clinical Hematology*, 12th ed. Baltimore: Lippincott Williams and Wilkins; 2009:1185–95.
2. Issaragrisil S, Leaverton PE, Chansung K et al. Regional patterns in the incidence of aplastic anemia in Thailand. The Aplastic Anemia Study Group. *Am J Hematol*. 1999;61:164–68.
3. Shimamura A, Alter B. Pathophysiology and management of inherited bone marrow failure syndromes. *Blood Rev*. 2010;24:101–22.
4. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood*. 2006;108:2509–19.
5. Young NS, Bacigalupo A, Marsh JCW. Aplastic anemia: pathophysiology and treatment. *Biol Blood Marrow Transplant*. 2010; 16:S119–25.
6. Marsh JC. Results of immunosuppression in aplastic anaemia. *Acta Haematol*. 2000;103:26–32.
7. Young NS. Hematopoietic cell destruction by immune mechanisms in acquired aplastic anemia. *Semin Hematol*. 2000;37:3–14.
8. Young NS, Kaufman DW. The epidemiology of acquired aplastic anemia. *Haematol*. 2008;93(4):489–92.
9. Calado RT, Young NS. Telomere maintenance and human bone marrow failure. *Blood*. 2008;111(9):4446–55.
10. Issaragrisil S, Kaufman DW, Anderson T et al. The epidemiology of aplastic anemia in Thailand. *Blood*. 2006;107(4):1299–1307.
11. Calado RT, Garcia AB, Gallo, DA et al. Reduced function of the multidrug-resistance P-glycoprotein in CD 34+ cells of patients with aplastic anemia. *Br J Haematol*. 2002;118:320–26.
12. Brown KE, Tisdale J, Barrett AJ et al. Hepatitis-associated aplastic anemia. *N Engl J Med*. 1997;336(15):1059–64.
13. Baker RI, Manoharan A, de Luca E et al. Pure red cell aplasia of pregnancy:a distinct clinical entity. *Br J Haematol*. 1993;85:619–22.
14. Young NS, Scheinberg P, Calado RT. Aplastic anemia. *Curr Opin Hematol*. 2008;15:162–68.
15. Socie G, Rosenfeld S, Frickhofen N et al. Late clonal diseases of treated aplastic anemia. *Semin Hematol*. 2000;37:91–101.
16. Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: recent progress. *Blood*. 2006;107(11):4223–33.
17. Alter BP. Bone marrow failure: a child is not just a small adult but an adult can have a childhood disease. *Hematology*. 2005;2005:96–103.
18. Marsh JCW, Ball SE, Cavenaugh J et al. Guidelines for the diagnosis and management of aplastic anemia. *Brit J Haematol*. 2009;147:43–70.
19. Valdez JM, Scheinberg P, Nunez O et al. Decreased infection-related mortality and improved survival in severe aplastic anemia in the past two decades. *Clin Infect Dis*. 2011;52:726–35.
20. Armand P, Antin JH. Allogeneic stem cell transplantation for aplastic anemia. *Biol Blood Marrow Transplant*. 2007;13:505–16.
21. Dessypris EN, Lipton JM. Red cell aplasia. In: Greer JP, Foester J, Rodgers GM et al., eds *Wintrobe's Clinical Hematology*, 12th ed. Baltimore: Lippincott Williams and Wilkins; 2009:1196–211.
22. Sawada K, Hirokawa M, Fujishima N. Diagnosis and management of acquired pure red cell aplasia. *Hematol Oncol Clin N Am*. 2009;23;249–59.
23. Hocking WG. Hematologic abnormalities in patients with renal diseases. *Hematol Oncol Clin North Am*. 1987;1:229–60.
24. Means RT. Anemias secondary to chronic disease and systemic disorders. In: Greer JP, Foester J, Rodgers GM et al., eds. *Wintrobe's Clinical Hematology*, 12th ed. Baltimore: Lippincott, Williams & Wilkins; 2009:1196–211.



17 Hemolytic Anemia: Membrane Defects

DIANA COCHRAN-BLACK, DrPH

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. List the hereditary membrane disorders involved with erythrocyte skeletal protein abnormalities.
2. List the hereditary erythrocyte membrane disorders involved with abnormal membrane permeability.
3. Describe the pathophysiology and recognize laboratory features associated with hereditary spherocytosis, hereditary elliptocytosis, hereditary pyropoikilocytosis, and hereditary stomatocytosis.
4. Discuss the principle of the osmotic fragility test and interpret the results.
5. Describe the etiology, pathophysiology, and laboratory features of paroxysmal nocturnal hemoglobinuria (PNH).
6. Explain the principle of the sugar water (sucrose hemolysis) test and interpret its results.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Differentiate the protein defects associated with each hereditary membrane defect discussed in the chapter.
2. Create a flow chart using laboratory tests to differentiate hereditary spherocytosis, elliptocytosis, pyropoikilocytosis, stomatocytosis, and paroxysmal nocturnal hemoglobinuria.
3. Explain the role of decay-accelerating factor (DAF) and membrane inhibitor of reactive lysis (MIRL) in PNH.
4. Explain the results of the sucrose hemolysis test and immunophenotyping to determine a diagnosis of paroxysmal nocturnal hemoglobinuria (PNH).
5. Evaluate a clinical case study and determine the type of membrane disorder present by correlating clinical history and laboratory features.

Chapter Outline

Key Terms

Abetalipoproteinemia
Cholecystitis
Cholelithiasis
Decay-accelerating factor (DAF)
Dehydrated hereditary stomatocytosis (DHS)
Exchange transfusion
Hereditary elliptocytosis (HE)
Hereditary pyropoikilocytosis (HPP)
Hereditary spherocytosis (HS)
Horizontal interactions
Membrane inhibitor of reactive lysis (MIRL)
Overhydrated hereditary stomatocytosis (OHS)
Paroxysmal nocturnal hemoglobinuria (PNH)
Rh null disease
Spur cell anemia
Vertical interaction

Background Basics

The information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the function, composition, and metabolism of the erythrocyte membrane. (Chapter 5)
- Identify the differences between extravascular and intravascular erythrocyte destruction. (Chapters 6, 11)
- Describe the general clinical and laboratory features associated with hemolytic anemias. (Chapter 11)
- Calculate erythrocyte indices, recognize abnormal erythrocyte morphology, and list reference intervals for common hematology parameters. (Chapters 10, 11, 37)

Level II

- List the erythrocyte membrane proteins associated with cell deformability and permeability, and describe their involvement in horizontal and vertical interactions. (Chapter 5)

CASE STUDY

We will refer to this case study throughout the chapter.

Jack, a 12-year-old male, was brought to his family physician for evaluation of right-upper-quadrant pain. He has a lifelong history of hemolytic and aplastic crises.

Consider why this patient history is important when selecting and evaluating laboratory tests.

OVERVIEW

This chapter focuses on a group of hemolytic anemias that result from defects in the erythrocyte membrane. These defects include hereditary spherocytosis, hereditary elliptocytosis, hereditary pyropoikilocytosis, and hereditary stomatocytosis; membrane lipid disorders; and paroxysmal nocturnal hemoglobinuria. The format for presentation of each of these disorders is pathophysiology, clinical features, laboratory findings, and therapy. Because these anemias result from defects in the erythrocyte membrane, the reader should have a good understanding of the normal erythrocyte membrane structure (Chapter 5) before beginning.

INTRODUCTION

Erythrocyte life span can be significantly shortened if the cell is intrinsically defective (intracorpuscular defect). Hemolytic anemia has been associated with defective erythrocyte membranes, structurally abnormal hemoglobins (hemoglobinopathies), defective globin synthesis (thalassemias), and deficiencies of erythrocyte enzymes. Almost all of these defects are hereditary. The hemoglobinopathies and thalassemias, which have a significant hemolytic component, are discussed in Chapters 13 and 14.

An erythrocyte membrane that is normal in both structure and function is essential to the survival of the cell (Chapter 5). Composed of proteins and lipids, the membrane is responsible for maintaining stability and the normal discoid shape of the cell, preserving cell deformability, and retaining selective permeability.

MEMBRANE DEFECTS

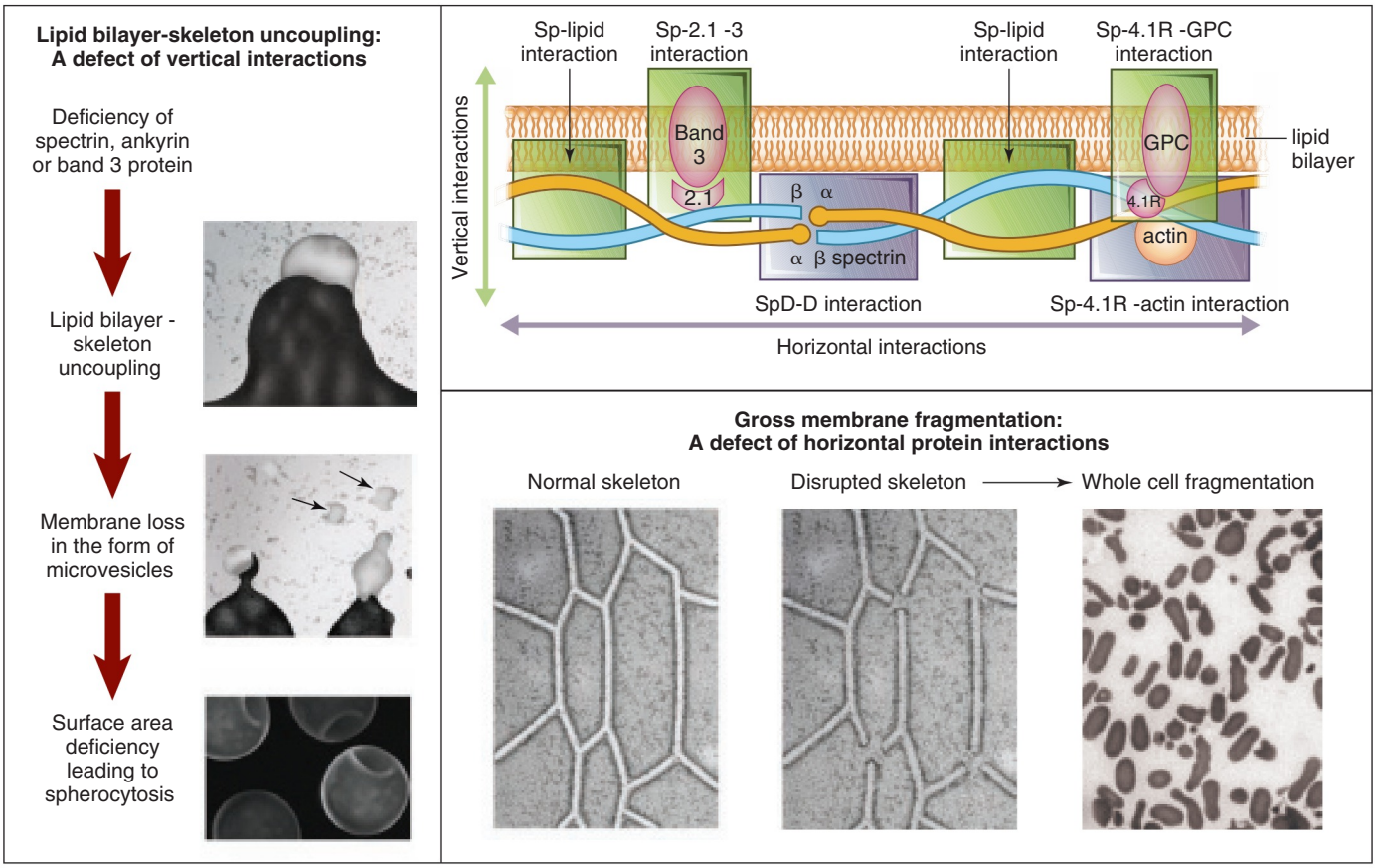
Hemolytic anemia can result from abnormalities in constituent membrane proteins or lipids, both of which can alter the membrane's stability, shape, deformability, and/or permeability. The abnormal cells are particularly susceptible to entrapment in the splenic cords. Anemia results when the rate of hemolysis is increased to the point that the bone marrow cannot adequately compensate. Most hemolysis associated with abnormal membranes is extravascular, occurring primarily in the spleen.

Skeletal Protein Abnormalities

The membrane protein and lipid interactions associated with abnormal erythrocyte membranes can be divided into two categories, vertical and horizontal interactions[1] (Figure 17-1 ■; Table 17-1 ★).

Vertical Interactions

Vertical interactions are perpendicular to the plane of the erythrocyte membrane and include interactions between the skeletal lattice on the cytoplasmic side of the membrane and its attachment to the integral proteins and lipids of the membrane. These interactions stabilize the lipid bilayer of the membrane. Defects in vertical contacts between the skeletal lattice proteins and the membrane's integral proteins and lipids cause uncoupling of the lipid bilayer from the underlying skeletal lattice, allowing a selective loss of portions of the lipid bilayer. The net loss of cell membrane results in a decrease in the surface-area-to-volume ratio, the formation of a spherocyte, and the eventual hemolysis of the cell. The skeletal lattice, however, is not



■ **FIGURE 17-1** Pathobiology of the red cell lesions in hereditary red blood cell membrane defects. Vertical interactions of membrane lipids and proteins are perpendicular to the plane of the membrane. These interactions stabilize the lipid bilayer. Deficiencies of spectrin, ankyrin (2.1), or band 3 protein cause the lipid bilayer to decouple from the underlying skeletal lattice and subsequent membrane loss in the form of microvesicles. This leads to the formation of spherocytes (hereditary spherocytosis). Horizontal interactions are parallel to the plane of the membrane. These interactions provide mechanical stability to the membrane. Horizontal defects include abnormal spectrin heterodimer association to form tetramers and defective skeletal protein interactions of junctional complexes at the end of the spectrin tetramers (spectrin, actin, protein 4.1R, previously referred to as protein 4.1). Horizontal defects result in fragmentation of the red blood cell (hereditary elliptocytosis, hereditary pyropoikilocytosis).

Sp = spectrin; GP = glycophorin; SpD-D = spectrin dimer dimer

★ **TABLE 17-1** Protein Mutations in the Erythrocyte Membrane That Result in Vertical or Horizontal Interaction Defects and Cause Hemolytic Anemia

Interaction Defect	Causes	Gene
Vertical	Ankyrin	*ANK1*
	Band 3	*SLC4A1*
	Protein 4.2	*EPB42*
	α-Spectrin	*SPTA1*
	β-Spectrin	*SPTB*
Horizontal	Protein 4.1R	*EPB41*
	Glycophorin C	*GYPC*
	α-Spectrin	*SPTA1*
	β-Spectrin	*SPTB*
	Actin	*ACTB*

disrupted, and the cell is mechanically stable. See Table 17-1 for the genetic defects associated with the vertical protein interactions of the red cell membrane.

Horizontal Interactions

Horizontal interactions are parallel to the plane of the membrane and are important in the formation of the stress-supporting skeletal protein lattice that provides mechanical stability to the membrane. Horizontal interactions include spectrin heterodimer head-to-head association to form tetramers as well as skeletal protein interactions in the junctional complexes at the distal ends of spectrin tetramers (spectrin, actin, protein 4.1R, glycophorin C, and adducin contacts). Horizontal defects characterized by defects of the skeletal protein interactions beneath the lipid bilayer lead to disruption of the skeletal lattice and, consequently, membrane destabilization. This causes cell fragmentation with formation of poikilocytes.

Lipid Composition Abnormalities

Disorders that affect the composition of the membrane lipid bilayer lead to the formation of acanthocytes or stomatocytes. The erythrocyte membrane normally contains equal amounts of free cholesterol and phospholipids. Excess free plasma cholesterol in patients can accumulate in the outer bilayer of the erythrocyte. Preferential expansion of the outer face of the lipid bilayer in comparison to the inner face leads to formation of acanthocytes.[2] Acanthocytes are more spheroidal cells with irregular projections and an absence of central pallor. These cells are poorly deformable and readily trapped in the spleen. A variety of conditions can lead to acanthocytosis.

Hemolytic anemias associated with the various membrane defects are listed in Table 17-2 ★. In general, vertical interaction defects are characterized by the presence of spherocytes; horizontal defects are characterized by the presence of other types of poikilocytes; and lipid composition defects are characterized by the presence of acanthocytes.

HEREDITARY SPHEROCYTOSIS

Hereditary spherocytosis (HS) is a common inherited membrane disorder that affects 1 in 2000 Caucasians. It is less common in African Americans and southeast Asians.[3] In about 75% of patients, the disease is inherited in an autosomal-dominant fashion, and recessive inheritance and de novo mutations account for the rest.[4]

CASE STUDY *(continued from page 318)*

A CBC was ordered on Jack, and the results follow:

WBC	8.0×10^9/L
RBC	4.0×10^{12}/L
Hb	108 g/L
Hct	0.292 L/L
Platelets	504×10^9/L

1. Calculate the erythrocyte indices.
2. Based on the calculated indices, describe the patient's red blood cells.

Pathophysiology

Hereditary spherocytosis is a clinically heterogeneous disorder characterized by mild to moderate hemolysis. The erythrocytes in this disorder are deficient in certain membrane proteins and are abnormally permeable to monovalent cations. These features lead to erythrocytes that have problems with both deformability and permeability. The intrinsic red cell defect is compounded by the function of the spleen, which retains and further damages the abnormal HS erythrocytes.

The membrane defect in HS is a disorder of vertical protein interactions most often characterized by a combined deficiency of spectrin and ankyrin, although there is considerable genetic heterogeneity at

★ **TABLE 17-2** Hemolytic Anemias Associated with Erythrocyte Membrane Defects

Disorder	Inheritance Pattern	Membrane Defect	Abnormal Membrane Function	Erythrocyte Morphology
Hereditary spherocytosis (HS)	Usually autosomal dominant; autosomal recessive; de novo mutations	Combined deficiencies of Ankyrin and spectrin or isolated deficiencies of spectrin, band 3, or protein 4.2	Defective vertical protein interaction between RBC skeleton and membrane; loss of lipid bilayer and subsequent formation of a spherocyte with decreased deformability; abnormal permeability to Na^+	Spherocytes
Hereditary elliptocytosis (HE)	Autosomal dominant or rare de novo mutation; Melanesian variant is autosomal recessive	Defective spectrin Deficiency or defect in band 4.1R Abnormal integral proteins	Defect in horizontal protein interactions resulting in membrane instability; also can be a defect in permeability	Elliptocytes
Hereditary pyropoikilocytosis (HPP)	Autosomal recessive	Two defects: Partial spectrin deficiency and presence of a mutant spectrin	Defect in horizontal protein interactions resulting in membrane instability	Schistocytes, elliptocytes, and microspherocytes
Overhydrated hereditary stomatocytosis	Autosomal dominant	Unknown	Abnormally permeable to Na^+ and K^+; overhydration and swelling; decreased deformability	Stomatocytes
Dehydrated hereditary stomatocytosis	Autosomal dominant	Unknown	Abnormally permeable resulting in loss of K^+; water loss; decreased deformability	Target cells
Acanthocytosis (abetalipoproteinemia)	Autosomal recessive	Increased sphingomyelin, which can be secondary to abnormal plasma lipid composition	Expansion of outer lipid layer causes abnormal shape; increased membrane viscosity and decreased fluidity/deformability	Acanthocytes
Paroxysmal nocturnal hemoglobinuria (PNH)	Acquired	Deficiency of DAF and MIRL	Increased sensitivity to complement lysis	Normocytic or macrocytic; microcytic, hypochromic if iron deficient

DAF = decay-accelerating factor; MIRL = membrane inhibitor of reactive lysis

the molecular level.[5,6] The spectrin deficiency can be a primary deficiency of spectrin or a secondary deficiency due to defective attachment of the skeleton to the lipid bilayer. Defective attachment can occur as a result of mutations of ankyrin, α- or β-spectrin, protein 4.2, or band 3 protein.[1,3,7] Identified mutations can be found at www.hgmd.cf.ac.uk/ac/index.php.

These defects in spectrin and ankyrin and their interactions with other skeletal proteins result in a weakening of the vertical connections between the skeletal proteins and lipid bilayer of the membrane. The uncoupling between the inner membrane skeleton and outer lipid bilayer leads to the shedding of the lipid bilayer in the form of lipid microvesicles.[3] Secondary to membrane loss, the cell has a decreased surface-area-to-volume ratio, changing the morphology of the cell from a discocyte to a spherocyte. The most spheroidal cells have a greatly increased cytoplasmic viscosity. The spheroidal shape and increased viscosity result in reduced cellular flexibility. Reticulocytes and young erythrocytes in HS are normal in shape, emphasizing the fact that erythrocytes lose their membrane fragments after encountering the stress of the circulation.

In addition to the abnormal cytoskeleton of HS erythrocytes, other membrane abnormalities can be present. Total lipid content in the HS erythrocyte membrane is decreased both before and after splenectomy. Although the organization of lipids in the membrane is known to affect membrane fluidity, an association between abnormal fluidity and HS erythrocytes has not been established. The HS erythrocytes also are abnormally permeable to sodium causing an influx of Na^+ at a much higher rate than normal.[8] An increase in the activity of the cation pump can compensate for the leak if adequate glucose is available for ATP production. The increased permeability is probably related to a functional abnormality of the membrane proteins.

The spherocytic shape, increased cytoplasmic viscosity, and increased membrane permeability account for the eventual destruction of HS cells in the spleen. The nondeformable spherocytes lack the flexibility of normal cells and become trapped in the splenic cords. In this acidic, hypoxic, hypoglycemic environment, the cell quickly runs out of the ATP needed to pump out the excess Na^+ resulting from the increased membrane permeability. As energy production decreases, splenic macrophages destroy the metabolically stressed cells.

Clinical Findings

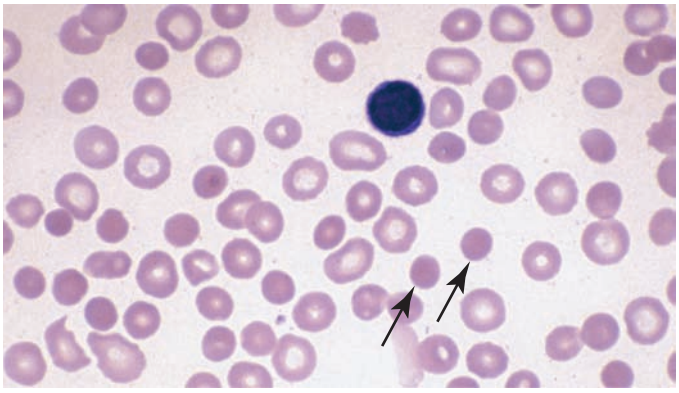
The clinical severity of HS varies among families and even among patients in the same family. About 25% of the patients have compensated hemolytic disease, no anemia, little or no jaundice, and only slight splenomegaly. In contrast, the disease can be lethal for some patients, especially in those homozygous for dominant HS. Most patients, however, develop a partially compensated hemolytic anemia in childhood and appear asymptomatic. HS in some asymptomatic individuals can be detected only when family studies are conducted on patients with more severe forms of the disease. Intermittent jaundice can occur and is especially apparent during viral infections. Splenomegaly is present in about 50% of affected infants increasing to 75–95% in older children and adults. Aplastic crisis is a life-threatening complication that can occur in childhood during or following a viral infection. Untreated older patients commonly develop pigment bile stones from excess bilirubin catabolism (**cholelithiasis**). These patients also are predisposed to **cholecystitis**.

Laboratory Findings

Hemoglobin levels in patients with HS can be normal or decreased, (about two-thirds to three-fourths have compensated or partially compensated mild to moderate anemia), varying directly with the individual's age upon presentation of symptoms. Infants have the lowest values, 8–11 g/dL. Older children usually have concentrations above 10 g/dL. The reticulocyte count is usually >8% and often is disproportionately increased relative to the degree of anemia.[9] The diagnosis of hereditary spherocytosis is suspected when many densely stained spherocytic cells with a decreased diameter and increased polychromasia on the blood smear are found (Figure 17-2 ■). Small, dense microspherocytes with a decreased MCV and increased MCHC also can be found. The number of microspherocytes varies considerably, and these cells may not be prominent in 20–25% of patients. In mild forms of HS, the changes in erythrocyte morphology can be too subtle to detect even by experienced hematologists. Nucleated erythrocytes can be found in children with severe anemia.

When the inheritance pattern of HS cannot be established, HS must be distinguished from other conditions causing spherocytosis. Spherocytes also can be found in acquired immune hemolytic anemia (AIHA), but in HS, the spherocytes are more uniform in size and shape than in AIHA.

Erythrocyte indices are helpful in diagnosing HS. The MCV usually is normal or only slightly decreased (77–87 fL). If reticulocytosis is marked, the MCV can be increased. The MCH is normal, but the MCHC is generally more than 36 g/dL.[10] Spherocytes are the only erythrocytes with an increased MCHC. Modern blood analyzers utilizing laser or aperture impedance methodology are extremely sensitive in detecting erythrocytes with an MCHC of more than 41 g/dL (hyperhemoglobin).[11] These erythrocytes are typically spherocytes. This technology can detect even the mild forms of HS. Importantly, the indices can vary, depending on iron and folate stores. Folate frequently becomes depleted in chronic hemolytic states, resulting in macrocytosis (Chapter 15). Other markers of ongoing hemolysis may be present including increased serum bilirubin, increased lactic dehydrogenase (LD), and increased urine and fecal urobilinogen. Variable components of extravascular and intravascular hemolysis may be present including decreased haptoglobin.



■ **FIGURE 17-2** Peripheral blood from a patient with hereditary spherocytosis shows the presence of many densely staining spherocytes (arrows) (peripheral blood; Wright-Giemsa stain; 1000× magnification)

As in other hemolytic states, the bone marrow demonstrates normoblastic erythroid hyperplasia with an increase in storage iron.

☑ CHECKPOINT 17-1

List various factors related to changes in the erythrocyte that can lead to a decrease or increase in the MCV in hereditary spherocytosis.

Osmotic Fragility Test

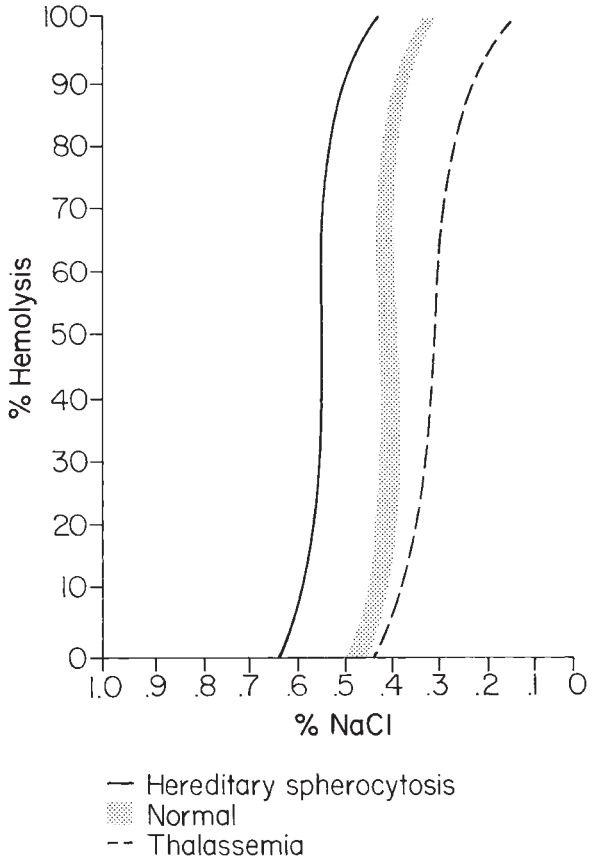
In many laboratories, the principal confirmatory test in the diagnosis of HS is the osmotic fragility test. This test measures the erythrocyte's resistance to hemolysis by osmotic stress, which depends primarily on the cell's volume, surface area, and its membrane function. Erythrocytes are incubated in varying concentrations of hypotonic sodium chloride (NaCl) solution. During the process of osmotic equilibration in hypotonic solutions, spherocytes are unable to expand as much as normal discoid shaped cells because of their decreased surface-area-to-volume ratio. Very little fluid needs to be absorbed before the cells hemolyze. The spherocytes also can have increased membrane permeability, contributing to their increased fragility. Lysis of HS erythrocytes, therefore, begins at higher NaCl concentrations than normal cells. These HS cells are said to exhibit increased osmotic fragility.

The osmotic fragility test is not abnormal unless spherocytes constitute at least 1–2% of the erythrocyte population; thus, patients with mild HS can have a normal osmotic fragility. These cells, however, show marked abnormal hemolysis if the blood is incubated overnight (24 hours) at 37°C before it is added to the NaCl solution. Incubation increases the loss of erythrocyte surface area in HS cells compared with normal erythrocytes due to their leaky and unstable membrane. The difference in osmotic fragility between HS cells and normal cells is particularly apparent at the 100% red cell lysis point which occurs at ~0.43 ± 0.05 g NaCl/dL with HS cells and at ~0.23 ± 0.07 g NaCl/dL with normal cells.[9] Because of its increased sensitivity, this incubated osmotic fragility test is the most reliable diagnostic test for hereditary spherocytosis.

The osmotic fragility test results are graphed to depict the degree of fragility in comparison to the normal state (Figure 17-3 ■). A shift to the left of normal in the curve indicates increased osmotic fragility, whereas a shift to the right indicates decreased osmotic fragility (Table 17-3 ★). Cells with an increased surface area–to–volume ratio (e.g., target cells) have a decreased osmotic fragility, which is found in conditions including thalassemia and sickle cell anemia.

Autohemolysis Test

The autohemolysis test also is used in diagnosing HS but does not have an advantage over the osmotic fragility test. It is more valuable in differentiating various types of congenital, nonspherocytic hemolytic anemias. This test measures the degree of spontaneous hemolysis of blood incubated at 37°C. The degree of hemolysis depends on the integrity of the cell membrane and the adequacy of cell enzymes involved in glycolysis. Incubation of blood in vitro probably causes an alteration of membrane lipids, which leads to a change in cell permeability and an increase in the utilization of both glucose and ATP.



■ **FIGURE 17-3** Graph depicting the osmotic fragility of normal cells, spherocytes, and cells from a patient with thalassemia. Spherocytes show an increased fragility with a shift to the left in the osmotic fragility curve, and the thalassemia cells show a decreased fragility with a shift to the right in the osmotic fragility curve. The decreased fragility in thalassemia is caused primarily by the presence of target cells.

In HS, autohemolysis is increased to between 5 and 25% at 24 hours (normal is 0.2–2.0%) and can increase to 75% at 48 hours. If glucose is added to the blood before incubation, hemolysis significantly decreases. If large numbers of microspherocytes are present, hemolysis might not be corrected with glucose. Autohemolysis also increases in immune spherocytic anemias, but glucose does not usually affect the test results in these conditions.

Antihuman Globulin Test

The antihuman globulin (AHG) test is negative, a finding helpful in distinguishing HS from immune hemolytic anemias in which large numbers of spherocytes also are found (Chapter 19). The AHG test detects antibodies or complement bound to erythrocytes in vivo

★ **TABLE 17-3** Example of Increased Osmotic Fragility

	Patient	Normal Control
Initial hemolysis	0.65% NaCl	0.45% NaCl
Complete hemolysis	0.45% NaCl	0.30% NaCl

(direct antiglobulin test, DAT) or free antibodies in the serum (indirect antiglobulin test, IAT). Immune hemolytic anemias are usually associated with a positive DAT whereas the DAT in HS is negative.

☑ CHECKPOINT 17-2

Explain why a patient with hereditary spherocytosis will demonstrate increased hemolysis in an autohemolysis test and why adding glucose prior to incubation will lead to a normal rate of hemolysis in these patients.

Identification of Deficient/Defective Membrane Protein

To determine which erythrocyte membrane protein(s) is/are involved, initial studies usually include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by densitometric quantitation and/or enzyme linked immunosorbent assay (ELISA) techniques.[10] Genetic linkage analysis using PCR-based techniques or cDNA and genomic DNA analyses can be done if a molecular diagnosis is needed (Chapter 42). Flow cytometric analysis of erythrocytes labeled with the fluorescent dye eosin-5-maleimide (EMA) has recently been introduced. The dye binds to the integral protein band 3 and Rh-related proteins in a 1:1 stoichiometry. Flow cytometry reveals a decrease in fluorescence in HS erythrocytes reflecting the relative amount of band 3. This test has high specificity and sensitivity for HS and is useful in screening.[9,12,13] The test is being offered by more laboratories as they become experienced with its use. The test also can detect other membrane abnormalities associated with band 3 including abnormalities of erythrocyte hydration, variants of dyserthropoietic anemia, and sickle cell disease.[9]

Therapy

Mild forms of HS do not require therapeutic intervention. Total or partial splenectomy is the standard treatment in patients with symptomatic hemolysis.[14,15] This therapy corrects the anemia and hemolysis, but the basic membrane defect remains, and spherocytes can still be found in the peripheral blood. Fragments of the unstable membrane are probably removed in the liver.

CASE STUDY *(continued from page 320)*

The patient's peripheral smear revealed numerous elliptocytes, spherocytes, teardrop cells, and micropoikilocytes.

3. What additional lab tests should be ordered?

HEREDITARY ELLIPTOCYTOSIS

Hereditary elliptocytosis (HE) is inherited as an autosomal-dominant trait except for a rare Melanesian type that is inherited as a recessive trait. Worldwide, the incidence is one case per 2000–4000 individuals, although the true incidence is unknown because many patients with HE are asymptomatic.[16] The disease is heterogeneous in the degree of hemolysis and in clinical severity. As its name indicates, the most prominent peripheral blood finding is an increase in oval and elongated erythrocytes (elliptocytes).

★ **TABLE 17-4** Classification of Hereditary Elliptocytosis (HE) Based on Erythrocyte Morphology

Type of HE	Hemolysis	Erythrocyte Shape
Common HE	Variable—minimal to severe	Biconcave elliptocytes
Spherocytic HE (hemolytic ovalocytosis)	Present	Spherocytes and fat elliptocytes
Southeast Asian ovalocytosis (SAO) (stomatocytic HE; Melanesian ovalocytosis)	Mild or absent	Roundish elliptocytes that are also stomatocytic

Three classifications of HE can be distinguished based on erythrocyte morphology[7]: (1) common HE, (2) spherocytic HE (hemolytic ovalocytosis), and (3) stomatocytic HE, Melanesian ovalocytosis, or Southeast Asian ovalocytosis (SAO) (Table 17-4 ★).

Pathophysiology

The abnormal erythrocyte shape in hereditary elliptocytosis is the result of a defect of one of the skeletal proteins. Similar to HS, reticulocytes and young erythrocytes in this disorder are normal in shape; therefore, the elliptical erythrocyte shape is acquired progressively as they circulate. Erythrocytes must deform (elongate) to enter capillaries in the microcirculation (vessels with diameters as small as ~3 mcM [μM]). Also, erythrocytes are subjected to shear stress as they circulate, which also contributes to their acquiring an elliptical shape. Normal erythrocytes revert to a biconcave shape once they re-enter larger vessels and experience less circulatory stress, whereas HE erythrocytes remain in the elliptical shape. It is possible that the stress in the microcirculation causes disruption of the skeletal protein contacts in the membrane of HE erythrocytes and leads to the formation of new protein contacts that prevent HE erythrocytes from resuming the normal biconcave shape.

The principal defect involves horizontal membrane protein interactions. Evidence indicates that several different membrane protein defects can be linked to this disease[17,18]:

1. Decreased association of spectrin dimers to form tetramers because of defective spectrin chains
2. Deficiency or defect in band 4.1 that aids in binding spectrin to actin
3. Abnormalities of the integral proteins including deficiency of glycophorin C and abnormal anion transport protein (band 3) with increased affinity for ankyrin (vertical interaction defect).

Each of these defects can lead to skeletal disruptions that can cause the cell to become elliptical in shape and/or fragment under the stresses of the circulation, depending on the extent of the defect. Cells become more elliptical as they age.[9] Mildly dysfunctional proteins cause only elliptocytosis, whereas severely dysfunctional proteins cause membrane fragmentation in addition to elliptocytosis. Alteration in shape only does not appear to affect cell deformability and viability, and the cells have a nearly normal life span. Elliptocytosis

with membrane fragmentation, however, causes a decrease in cell surface area and reduced cell deformability. The life span of these cells is severely shortened.

In addition to membrane instability, HE erythrocytes are abnormally permeable to cations. This altered permeability demands an increase in ATP to run the cation pump and maintain osmotic equilibrium. Cells detained in the spleen can quickly deplete their ATP and become osmotically fragile.

The defect in the SAO variant of HE is an abnormal band 3 protein rather than a defect in the cytoskeletal proteins under the lipid bilayer. There is a nine-amino acid deletion near the boundary of the membrane and cytoplasmic domains of the protein. This is associated with a tighter binding of band 3 to ankyrin, a lack of transport of sulfate anions, and a restriction in the lateral and rotational mobility of band 3 protein within the membrane. As a result, these erythrocytes are very rigid.[7]

Clinical Findings

Ninety percent of patients with HE show no overt signs of hemolysis. Although erythrocyte survival can be decreased, the hemolysis is usually mild and well compensated for by an increase in bone marrow erythropoiesis (compensated hemolytic disease). Anemia is not characteristic.

Common HE is rare in the Western populations but more common in blacks, particularly in equatorial Africa. The severity ranges from asymptomatic to severe clinical disease. There can be minimal hemolysis and only mild elliptocytosis (15%) or severe hemolysis with cell fragmentation and formation of poikilocytes.

A variant of common HE noted in black infants is associated with a moderately severe anemia at birth and neonatal jaundice. The peripheral blood smear exhibits erythrocytes similar to those seen in hereditary pyropoikilocytosis with budding and fragile bizarre poikilocytes. Variable numbers of elliptocytes are noted. Between 6 and 12 months of age, the hemolysis decreases, and the number of elliptocytes increases. One of the parents of affected infants has mild hereditary elliptocytosis.

The spherocytic HE variant constitutes a relatively rare form of HE characterized by the presence of hemolysis despite minimal changes in erythrocyte morphology. The erythrocytes have characteristics of both HS and HE cells: Some are spherocytic and others are fat elliptocytes.

The Southeast Asian variant of HE (SAO, stomatocytic HE) is characterized by a mild or absent hemolytic component. Erythrocyte cation permeability appears to be increased, and the expression of blood group antigens is muted. The elliptocytes are roundish and stomatocytic with one or two transverse bars or a longitudinal slit. Evidence indicates that these cells can have more stable cytoskeletons than normal.[17]

The high prevalence of the SAO variant of HE in some parts of the world is related to the resistance of the SAO erythrocytes to invasion by malarial parasites. The resistance may result from the abnormal rigidity of the erythrocyte membrane. This protection against malaria has led to natural selection of individuals with HE in areas of the world where malaria is endemic.[7]

HE cells are poorly agglutinable with antisera against erythrocyte antigens. This is presumably the result of defective lateral movement and clustering of surface antigens associated with the abnormal band 3 protein. The laboratory scientist should be aware of this problem because it can interfere with testing of patients' cells in the blood bank.

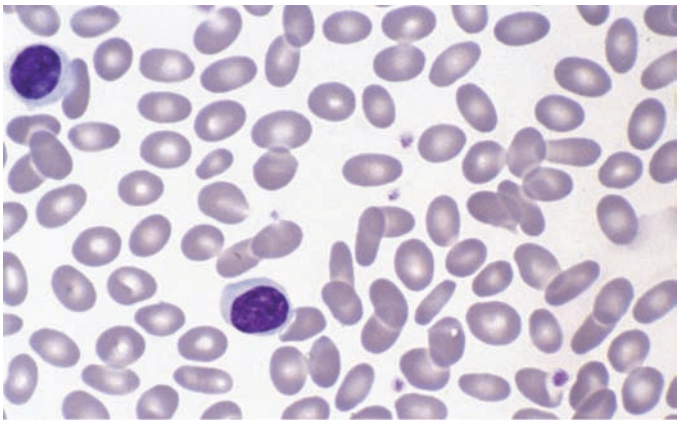


FIGURE 17-4 Peripheral blood from a patient with nonhemolytic HE reveals almost 100% elliptocytes (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Laboratory Findings

The most consistent and characteristic laboratory finding in all variants is prominent elliptocytosis (Figure 17-4 ■). Elliptocytes also can be found in association with other diseases, but elliptocytes in these conditions usually constitute less than 25% of the erythrocytes. Acquired elliptocytosis is seen in megaloblastic anemias and iron-deficiency anemia. In contrast, the elliptocytes in HE make up more than 25% of the erythrocytes and usually more than 60%. In the asymptomatic variety of HE, elliptocytes could be the only morphologic clue to the disease. Hemoglobin levels are usually higher than 12 g/dL. Reticulocytes are mildly elevated, up to about 4%.

In the hemolytic HE variants, hemoglobin concentration is 9–10 g/dL, and reticulocytes are elevated to as high as 20%. Microelliptocytes, bizarre poikilocytes, schistocytes, and spherocytes are usually evident (Figure 17-5 ■). The bone marrow shows erythroid hyperplasia with normal maturation.

The incubated and unincubated osmotic fragility tests and autohemolysis tests are usually abnormally increased in the overt hemolytic variants. However, the obvious blood picture suggests that there is no need to perform these tests.

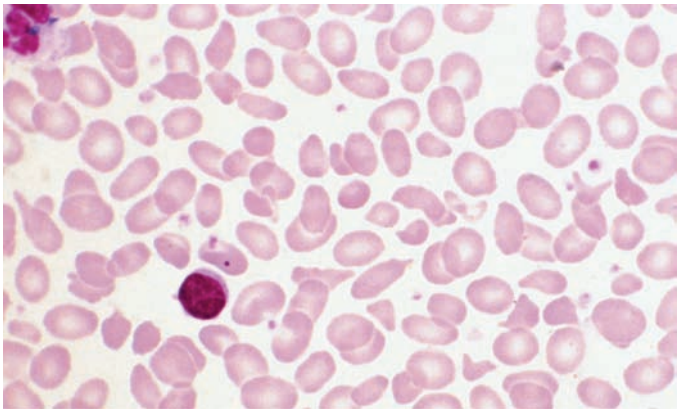


FIGURE 17-5 Peripheral blood from a patient with hemolytic hereditary elliptocytosis. Schistocytes as well as elliptocytes are present (Wright-Giemsa stain, 1000× magnification).

Therapy

The hemolytic variants of HE respond well to splenectomy. As in HS, splenectomy reduces hemolysis and protects the patient from complications of chronic hemolysis. The membrane defect, however, remains, and elliptocytes are still present. The asymptomatic variants require no therapy.

☑ CHECKPOINT 17-3

Why do the elliptocytes in HE demonstrate normal osmotic fragility?

CASE STUDY (continued from page 323)

Jack's osmotic fragility test revealed the following:

	Patient	Control
Initial hemolysis	0.65% NaCl	0.45% NaCl
Complete hemolysis	0.40% NaCl	0.30% NaCl

4. Interpret the results of the osmotic fragility test.

HEREDITARY PYROPOIKILOCYTOSIS (HPP)

Hereditary pyropoikilocytosis (HPP), a rare autosomal recessive disorder, is closely related to HE. Based on genetic and biochemical data, it has been established that HPP is a severe subtype of HE.[16,18] It occurs primarily in blacks, although it also has been diagnosed in Arabs and Caucasians.[2] The disease presents in infancy or early childhood as a severe hemolytic anemia with extreme poikilocytosis. The morphological similarities of erythrocytes in HPP and that of erythrocytes associated with thermal injury led investigators to examine the thermal stability of HPP cells. In contrast to normal erythrocytes that fragment at 49–50°C, HPP cell membranes fragment when heated to 45–46°C. In addition, pyropoikilocytes disintegrate when incubated at 37°C for >6 hours.

Pathophysiology

The HPP cells have two defects with one inherited from each parent. One is related to a deficiency of α-spectrin and the other to the presence of a mutant spectrin that prevents self-association of heterodimers to tetradimers.[17] The parent carrying the mutant spectrin either has mild HE or is asymptomatic. The parent with the deficiency of spectrin, is usually hematologically normal. The HPP phenotype also is found in patients who are homozygous or doubly heterozygous for one or two spectrin mutations characteristically found in HE trait.[17] These defects lead to a disruption of the membrane skeletal lattice and cell destabilization followed by erythrocyte fragmentation and poikilocytosis. Poikilocytes are removed in the spleen.

Clinical Findings

Clinical features consistent with a hemolytic anemia are present at birth. Hyperbilirubinemia requiring **exchange transfusion** (a procedure involving simultaneous withdrawal of blood and infusion with compatible blood) or phototherapy (therapeutic exposure to sunlight or artificial light) is present.[17] Laboratory serologic studies for hemolytic disease of the newborn, however, are negative.

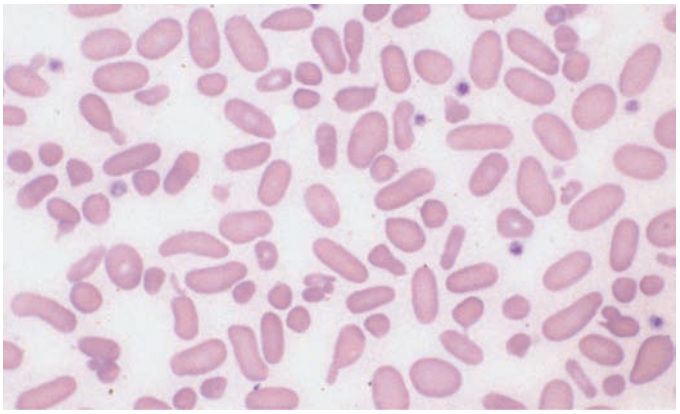


FIGURE 17-6 Peripheral blood from a patient with hereditary pyropoikilocytosis (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Laboratory Findings

Stained blood smears exhibit striking erythrocyte morphologic abnormalities including budding, fragments, microspherocytes (2–4 fL), elliptocytes, triangulocytes (fragmented erythrocytes that are triangle shaped), and other bizarre erythrocyte shapes (Figure 17-6 ■). The MCV is decreased (25–55 fL), most likely as a result of the erythrocyte fragmentation. The osmotic fragility is abnormal, especially after incubation, and the thermal sensitivity test demonstrates an increase in erythrocyte fragmentation (Figure 17-7 ■). Autohemolysis is increased, and the hemolysis is not corrected with glucose.

Therapy

Patients show improvement after splenectomy, but the membrane defect remains, and fragmented erythrocytes are still present.

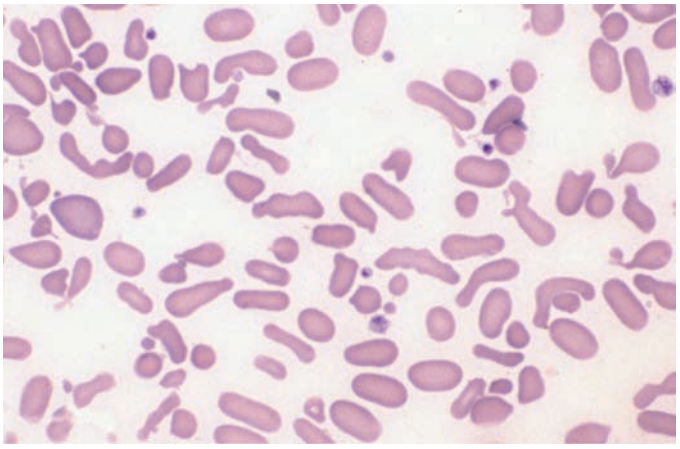


FIGURE 17-7 Peripheral blood from a patient (same as in Figure 17-6) with hereditary pyropoikilocytosis. Smear made after incubation of blood for 10 minutes at 46°C (peripheral blood, Wright-Giemsa stain, 1000× magnification).

☑ CHECKPOINT 17-4

Interpret the results of the following thermal sensitivity test:

Patient's erythrocytes	Marked erythrocyte fragmentation after 10-minute incubation at 46°C
Normal control	No significant change in erythrocyte morphology after 10-minute incubation at 46°C.

HEREDITARY STOMATOCYTOSIS SYNDROMES

The term *hereditary stomatocytosis* includes a group of rare autosomal dominant hemolytic anemias in which the erythrocyte membrane exhibits abnormalities in cation permeability.[8] These syndromes include **overhydrated hereditary stomatocytosis (OHS)** (also known as *hereditary hydrocytosis*) and **dehydrated hereditary stomatocytosis (DHS)** (also known as *hereditary xerocytosis*). The red cell membrane in OHS is abnormally permeable to both Na^+ and K^+. The net gain of Na^+ ions is greater than the net loss of K^+ ions as the capacity of the cation pump (fueled by ATP derived from glycolysis) to maintain normal intracellular osmolality is exceeded. Because the pump exchanges 3 Na^+ (outward) for 2 K^+ (inward), as the pump fails, the intracellular concentration of cations increases, water enters the cell, and the overhydrated cells take on the appearance of stomatocytes. Stomatocytes (hydrocytes) on dried, stained blood films are erythrocytes with a slitlike (mouthlike) area of pallor (Figure 17-8 ■). These cells are uniconcave and appear to be bowl shaped on wet preparations.

The primary defect in DHS is a net loss of intracellular K^+ that exceeds the passive Na^+ influx, and net intracellular cation and water content are thus decreased. Consequently, the cell dehydrates and the cells appear targeted or contracted and spiculated.

CASE STUDY (continued from page 325)

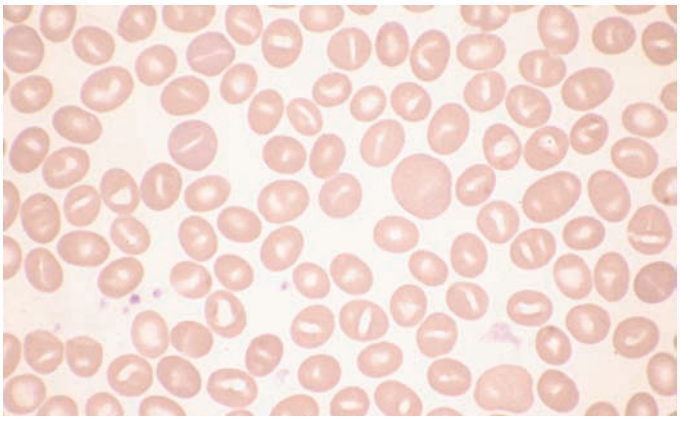
A thermal sensitivity test demonstrated that Jack's erythrocytes fragment when incubated for 10 minutes at 46°C.

5. What do the results of the thermal sensitivity test reveal about Jack's red blood cells?

Pathophysiology

The specific membrane defect for OHS and DHS has not been identified. Patients with OHS demonstrate a deficiency of stomatin (band 7.2b), an integral red cell membrane protein, which is secondary to a yet to be found protein mutation.[2,19] Abnormalities of erythrocyte lipids have been demonstrated to induce stomatocytosis with impaired sodium transport. However, stomatocytosis also occurs when membrane lipids are normal. In these cases, membrane proteins can be abnormal. Although membrane proteins are usually electrophoretically normal, their conformation may be altered.

From the variability of clinical findings, laboratory results, and response to splenectomy, these disorders appear to be caused by several different membrane defects. Some patients, have marked stomatocytosis but no abnormal sodium transport and, thus, no overt hemolysis. **Rh null disease** (a disorder associated with the lack of all Rh antigens on erythrocytes) also is associated with the presence of stomatocytes. Rh proteins are normally noncovalently linked with Rh-associated glycoproteins (RhAG) and together they form the Rh–RhAG complex. A lack of this complex alters the cation regulation of the erythrocyte.[20,21]



■ **FIGURE 17-8** This peripheral blood picture from a patient with hereditary overhydrated stomatocytosis reveals erythrocytes with slitlike or mouthlike (stoma = mouth) areas of pallor (peripheral blood, Wright-Giemsa stain, 1000× magnification).

The stomatocytic cells in OHS are osmotically fragile and less deformable than normal cells, and, as a result, the cells are sequestered in the spleen where glucose supplies are readily exhausted. As the ATP levels fall, the cation pump is unable to maintain osmotic equilibrium, causing cell lysis or phagocytosis.

OHS must be differentiated from acquired stomatocytosis. Stomatocytes are seen as an acquired defect in acute alcoholism, liver disease, and cardiovascular disease. However, these acquired conditions lack abnormal cation permeability, and little hemolysis is present.

The cells are dehydrated in DHS, which is reflected by an increased MCHC. As the MCHC of the cell increases beyond 37 g/dL, the cytoplasmic viscosity increases, and cellular deformability decreases. The rigid cells become trapped in the spleen.

Laboratory Findings

Anemia is usually mild to moderate with a hemoglobin concentration of 8–10 g/dL. Bilirubin is increased and reticulocytosis is moderate. The MCHC of the stomatocytes seen in OHS is decreased, and the MCV can be increased. The blood smear is remarkable for 10–50% stomatocytes. Osmotic fragility and autohemolysis are increased, and autohemolysis is partially corrected with glucose and ATP.

Target cells and erythrocytes that have hemoglobin puddled at the periphery are observed in DHS. The cells demonstrate a slightly increased MCV, increased MCHC, and decreased osmotic fragility.

☑ CHECKPOINT 17-5

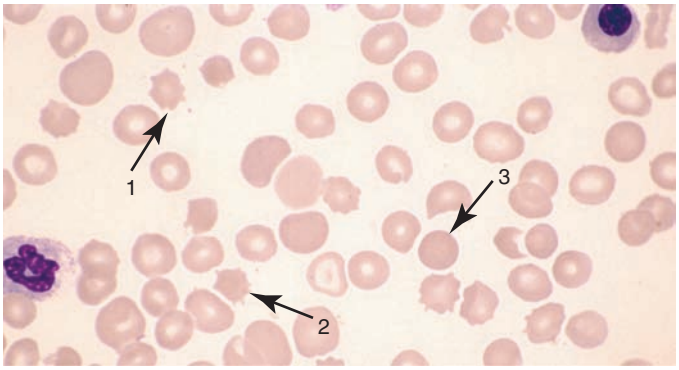
Explain why the osmotic fragility is decreased in DHS.

Therapy

Splenectomy is not a required therapeutic measure for OHS and DHS. In fact, this procedure is usually contraindicated because some patients have experienced catastrophic thrombotic episodes after splenectomy.[20,22] Most patients are able to maintain an adequate hemoglobin level without this procedure.

☑ CHECKPOINT 17-6

Determine the type of erythrocyte membrane disorder present based on the following lab results: reticulocyte count: 4%; osmotic fragility: increased; autohemolysis: increased; bilirubin: increased; peripheral smear: 30% stomatocytes present.



■ **FIGURE 17-9** Spur cell anemia in a patient with alcoholic cirrhosis. Note the echinocytes (1) and acanthocytes (spur cells; 2). Spherocytes (3) are also present (peripheral blood, Wright-Giemsa stain, 1000× magnification).

ABNORMAL MEMBRANE LIPID COMPOSITION: ACANTHOCYTOSIS

Acanthocytosis is most often associated with acquired or inherited abnormalities of the membrane lipids. This occurs in liver disease and in a rare inherited condition, *abetalipoproteinemia*. Although the mature erythrocyte has no capacity for de novo synthesis of lipids or proteins, the lipids of the membrane continually exchange with plasma lipids. Thus, erythrocytes can acquire excess lipids when the concentration of plasma lipids increases. Excess membrane lipids expand the membrane surface area and cause the cell to acquire abnormal shapes, including target cells (codocytes), leptocytes, or acanthocytes. If portions of the membrane are lost because of "grooming" of excess lipids in the spleen or if the membrane's lipid viscosity increases, the cells lose their ability to deform and are sequestered in the spleen. On the other hand, a significant decrease in one or more lipids in the cell membrane can also lead to increased destruction. The rare hereditary forms of acanthocytosis also can result from abnormal proteins.

Spur Cell Anemia

Spur cell anemia is an acquired hemolytic condition associated with severe hepatocellular disease such as cirrhosis in which serum lipoproteins increase, leading to an excess of erythrocyte membrane cholesterol. The total phospholipid content of the membrane, however, is normal. As the membrane ratio of cholesterol to phospholipid increases, the cell becomes flattened (leptocyte) with a scalloped edge. The increased cholesterol-to-phospholipid ratio also causes a decrease in membrane fluidity and an associated decrease in cell deformability. During repeated splenic passage or conditioning, membrane fragments are lost, and the cell acquires irregular spikelike projections typical of acanthocytes (spur cells). Eventually, the cell is hemolyzed. In patients with cirrhosis, this hemolysis is enhanced by congestive splenomegaly.

The peripheral blood shows a moderate to severe normocytic, normochromic anemia with a hemoglobin concentration of 5–10 g/dL. Reticulocytes are increased to 5–15%. Approximately 20–80% of the erythrocytes are acanthocytes (Figure 17-9 ■). On the peripheral blood smear, acanthocytes must be distinguished from echinocytes (burr cells) and keratocytes. Spherocytes and echinocytes can also be found. An increase in unconjugated bilirubin and liver enzymes and a decrease in serum albumin reflect evidence of liver disease.

Studies with transfused cells have shown that spur cells acquire their shape as innocent by-standers and that when normal cells are transfused into the patient, they acquire the abnormal shape and are hemolyzed at the same rate as the patient's cells. This suggests that the diseased liver and associated plasma lipid abnormalities are responsible for the transformation of normal red cells into spur cells.[23]

Biliary obstruction is often associated with the presence of normocytic or slightly macrocytic target cells that result from an acquired excess of lipids in the cell membrane. However, in contrast to the acanthocytes found in severe hepatocellular disease, the excess lipid in target cells associated with biliary obstruction includes an increase in both cholesterol and phospholipids in a ratio similar to that of normal cells. As a result, lipid viscosity and membrane deformability are normal. These target cells have a normal survival.

Abetalipoproteinemia (Hereditary Acanthocytosis)

Abetalipoproteinemia is a rare autosomal recessive disorder characterized by the absence of serum β-lipoprotein, low serum cholesterol, low triglyceride, and low phospholipid and an increase in the ratio of cholesterol to phospholipid.[24] The primary abnormality is the defective synthesis of apolipoprotein B, a microsomal triglyceride transfer protein. Mutations in band 3 protein of the erythrocyte membrane that alters band 3 conformation have also been described in hereditary acanthocytosis.[25] Acanthocytes are typically found, but hemolysis is minimal with little or no anemia. Reticulocytes are usually normal but can be slightly increased. The acanthocytes have normal cholesterol levels, but lecithin is decreased and sphingomyelin is increased. This is in contrast to spur cells found in severe liver disease that have increased membrane cholesterol. Membrane fluidity is decreased, presumably because of the increase in sphingomyelin, which is less fluid than other phospholipids. The degree of distortion of erythrocytes increases with cell age. The acanthocytes have

normal membrane permeability, normal glucose metabolism, and normal osmotic fragility. Autohemolysis at 48 hours is increased and only partially corrected by glucose. In addition to hypolipidemia, the disorder is characterized by steatorrhea, retinitis pigmentosa, and neurological abnormalities. Transport of fat-soluble vitamins (A, D, E, K) is impaired, and the prothrombin time can be increased because of decreased vitamin K stores (Chapters 32 and 36).

Lecithin-Cholesterol Acyl Transferase (LCAT) Deficiency

This rare autosomal recessive disorder affects metabolism of high-density lipoproteins. Onset is usually during young adulthood. The disorder is characterized by a deficiency of LCAT, the enzyme that catalyzes the formation of cholesterol esters from cholesterol. As a result, patients with this deficiency demonstrate low levels of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) and elevated levels of very low-density lipoproteins (VLDL) and lipoprotein X.[26] Because erythrocyte membrane cholesterol is in a relatively rapid equilibrium with unesterified plasma cholesterol, the activity of LCAT indirectly regulates the amount of free cholesterol in the cell. The most characteristic hematologic findings include a mild hemolytic anemia marked by the presence of numerous target cells. The target cells are loaded with cholesterol. LCAT activity decreases in most patients with spur cells and target cells, but the relationship to lipid abnormalities of the membrane is not known.

Rare Forms

The rare forms of acanthocytosis associated with abnormalities of membrane proteins include the McLeod phenotype with a deficiency of Kx protein and the K antigen, chorea-acanthocytosis syndrome, and acanthocytosis with band 3 protein abnormalities.

☑ CHECKPOINT 17-7

Compare the membrane lipid abnormalities seen in LCAT deficiency, spur cell anemia, and abetalipoproteinemia, and explain how they result in hemolysis of the cell.

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

All erythrocyte membrane disorders discussed so far are hereditary except spur cell anemia. **Paroxysmal nocturnal hemoglobinuria (PNH)** is a rare acquired disorder of the erythrocyte membrane. The membrane defect makes the cell abnormally sensitive to lysis by complement. The disease derives its name from the classic pattern of intermittent bouts of intravascular hemolysis and nocturnal hemoglobinuria. The condition is exacerbated during sleep and remits during the day. However, many patients have chronic hemolysis that is not associated with sleep and with no obvious hemoglobinuria.

Pathophysiology

An intrinsic erythrocyte disorder, PNH results from an acquired stem cell somatic mutation that leads to an abnormal clone of differentiated hematopoietic cells. The abnormal stem cell clone produces erythrocytes, platelets, and neutrophils that bind abnormally large amounts of complement and that are abnormally sensitive to complement lysis (Chapter 19). Complement is composed of at least 20 proteins responsible for a variety of biologic activities. These proteins are abbreviated as C1 through C9. They normally circulate in an inactive form and are activated as part of the body's defense system (Chapter 7). If activated complement components attach to the erythrocyte membrane, the cell can be hemolyzed.

The susceptibility of PNH cells to complement-induced lysis is related to deficient regulation of complement activation. At least two regulatory proteins, **decay-accelerating factor (DAF)** (CD55) and **membrane inhibitor of reactive lysis (MIRL)** (CD59), found on normal cell membranes are responsible for preventing amplification of complement activation. DAF is a complement regulatory protein that amplifies the decay of C3 convertase, accelerates decay (dissociation) of membrane-bound C3bBb, and thus prevents amplification of C3 convertase and activation of other complement components. The major role of MIRL is to prevent the interaction between C8 and C9; thus, it interferes with the formation of the membrane attack complex (MAC) (the final steps of complement activation). These regulatory factors help normal cells avoid lysis by autologous complement. Lack of DAF and MIRL on PNH cells is causally related to excessive sensitivity of these cells to complement.[27]

Deficiency of DAF and MIRL in PNH is not due to the lack of production of these proteins but to the absence of a membrane glycolipid that serves as an anchor that attaches these proteins to the cell membrane. Other proteins anchored to the cell membrane in a similar manner also are deficient on hematopoietic cells in PNH. The common link in the deficiencies of these membrane proteins is the lack of the glycolipid anchoring structure, glycosyl-phosphatidyl inositol (GPI). GPI embedded in the cell membrane is important for the covalent linkage of a wide variety of proteins to the cell membrane. These GPI-linked proteins vary in structure and function, and include adhesion molecules, hydrolases, and receptors.[27]

The GPI-anchoring deficiency is because of a somatic mutation of the *PIGA* gene, which encodes the enzyme phosphatidyl inositol glycan Class A essential for synthesis of the GPI anchor. A large number of *PIGA* mutations have been identified. Analysis of bone marrow cells indicates that the mutation occurs in the hematopoietic stem cell (HSC). Although not understood, the mutated HSC has a proliferative advantage. All blood cell lineages derived from the mutant HSC are deficient in DAF activity, confirming PNH as a clonal disorder of hematopoiesis. Thus, many patients with PNH are not only anemic but also granulocytopenic and thrombocytopenic. The abnormal clone can appear after damage to the marrow or spontaneously (idiopathic). A significant number of patients with PNH have or eventually develop another clonal blood disorder such as acute nonlymphocytic leukemia (Chapter 26), chronic lymphocytic leukemia (Chapter 28), myeloproliferative disorder (Chapter 24), or myelodysplastic syndrome (Chapter 25).[28]

The increased sensitivity of PNH cells to complement can be demonstrated in vitro by activation of either the classic or the alternate pathway of complement activation. In vivo, however, activation is probably primarily via the alternate pathway.

Clinical Findings

PNH occurs most often in adults but is occasionally found in children. The four basic disease manifestations are hyperhemolysis, venous thrombosis, infection, and bone marrow hypoplasia.[28] PNH begins insidiously with irregular brisk episodes of acute intravascular hemolysis accompanied by hemoglobinuria. The patient usually seeks medical attention when reddish-brown urine is noted. In some patients, the irregular exacerbations of hemolysis are associated with sleep, hence the name *paroxysmal nocturnal hemoglobinuria*. In other patients, these hemolytic episodes can follow infections, transfusions, vaccinations, surgery, or ingestion of iron salts. In a large number of patients, hemolysis is unrelated to any specific event. Iron-deficiency anemia can occur because of the chronic intravascular hemolysis and urinary loss of iron-containing degradation products. Renal function can become abnormal as the result of chronic iron deposition. Folic acid deficiency can occur because of increased demand for this nutrient. Abdominal and lower back pain, eye pain, and headaches can occur during hemolytic episodes.

In spite of the moderate thrombocytopenia, venous thrombosis is a prominent and severe complication and is a common cause of death. Thrombotic events may be related to abnormal platelet or neutrophil function because of the lack of GPI-anchored proteins.

When leukopenia is present, infections are common. Propensity for infection also can be related to the absence of granulocyte glycoproteins and altered functional responses of granulocytes. Immunological abnormalities can be present.

Laboratory Findings

Most patients with PNH experience anemia with a hemoglobin concentration of 8–10 g/dL. The erythrocytes are normocytic or macrocytic but can appear microcytic and hypochromic if iron deficiency develops. Reticulocytes are increased (5–10%) but not to the extent expected for a hemolytic anemia. Nucleated red blood cells can be found on the blood smear. Isolated development of leukopenia and/or thrombocytopenia often occurs during the course of the disease. Neutrophil alkaline phosphatase and erythrocyte acetylcholinesterase decrease (Chapter 23). Although hemoglobinuria can be intermittent or even mild, hemosiderinuria is a constant finding, indicating chronic intravascular hemolysis.

The bone marrow usually exhibits normoblastic hyperplasia but can be hypocellular. In some cases, marrow failure develops during the course of the disease. Interestingly, aplastic anemia can be the initial diagnosis with an abnormal clone of PNH cells developing during the course of the disease (in up to 50% of patients with aplastic anemia, Chapter 16). PNH rarely precedes aplastic anemia and should be considered as a diagnosis when hypoplastic anemia is found in association with hemolysis.[28] Bone marrow iron is decreased or absent.

The osmotic fragility is normal. Autohemolysis is increased after 48 hours, and when glucose is added, the hemolysis can increase even more. The DAT is negative for immunoglobulin (Ig) but can be positive for complement given the fact that PNH cells have a propensity to bind C3b.

The sucrose hemolysis (sugar-water) test is useful in identifying erythrocytes that are abnormally sensitive to complement lysis. The patient's blood is incubated in a sucrose solution. The sucrose provides a low-ionic-strength medium that promotes the binding of complement to the erythrocytes. PNH cells show hemolysis in this medium.

The Ham (acidified-serum lysis) test is a more specific test for PNH cells (Chapter 37), but laboratories have replaced it with immunophenotyping for confirmation of PNH (Chapter 40). Immunophenotyping uses monoclonal antibodies directed against the GPI anchored molecules. When the GPI link is missing, these molecules are also missing. This technology can detect three types of cells in PNH related to the degree of deficiency of GPI-linked proteins on cell membranes:

- **Type I** Little or no hemolysis by complement; nearly normal GPI-linked protein expression
- **Type II** Moderately sensitive to complement lysis; intermediate GPI-linked protein expression
- **Type III** Highly sensitive to complement lysis; no expression of GPI-linked proteins

Because different hematopoietic cells display different types of GPI-anchored proteins, it is recommended that at least two different antibodies be used for a diagnosis of PNH (CD55, CD59, CD14). CD55 (DAF) and CD59 (MIRL) antibodies are used most frequently and are most attractive because they are involved in the complement pathway that directly leads to the hemolysis in PNH. PNH cells show low-intensity staining for these molecules. CD55 and CD59 are both normally found on all hematopoietic cells. CD14 is normally on monocytes, and some laboratories use it to verify PNH. The analysis of molecules that can be expressed on the membrane in a GPI-anchored form as well as a transmembrane form is not as useful because it can give false normal results.

The most accurate measurement of the PNH defect with immunophenotyping is performed using nucleated cells rather than erythrocytes because the patient with PNH often receives red blood cell transfusions. In addition, results on erythrocytes might not be accurate because PNH type III erythrocytes have a shorter life span than do type I or II, thus underestimating type III erythrocyte concentration.

The fluorescent-labeled inactive toxin aerolysin (FLAER) test is another method used to diagnose PNH.[29] This technique utilizes toxin from the bacterium *Aeromonas hydrophila* bound to a fluorochrome. The toxin binds to the GPI anchor itself, not to an attached protein, which makes it a direct measurement of GPI-deficient cells.

☑ CHECKPOINT 17-8

Explain why immunophenotyping with CD14, CD55, and CD59 is used to establish a diagnosis of PNH.

Therapy

Treatment is primarily supportive in the form of transfusions, antibiotics, and anticoagulants. A humanized monoclonal antibody against C5 (eculizumab) has been shown to relieve the hemoglobinuria in PNH patients by inhibiting the formation of the membrane attack complex (MAC).[30] In patients with PNH-induced marrow aplasia, bone marrow transplantation may be indicated.

CASE STUDY *(continued from page 326)*

This case study depicts a young man who presented with right-upper-quadrant pain. The CBC disclosed anemia with a decreased MCV and increased MCHC. The peripheral blood smear revealed numerous elliptocytes, teardrop cells, and micropoikilocytes. The patient's erythrocytes demonstrated increased osmotic fragility and thermal sensitivity.

6. What disorder do the patient's lab findings suggest?

Summary

Intrinsic defects of the erythrocyte such as an abnormal membrane, structurally abnormal hemoglobin, defective globin synthesis, or deficient erythrocyte enzymes can lead to a shortened erythrocyte life span because of increased hemolysis. An anemia will result if the bone marrow is unable to compensate for the erythrocyte loss. These intrinsic abnormalities are almost always inherited defects.

Erythrocyte membrane defects can be caused by abnormalities of membrane proteins or lipids, which can affect cell deformability, stability, shape, and/or permeability. The abnormal erythrocytes become trapped in splenic cords and are removed from the circulation. Interactions between the skeletal lattice proteins and integral proteins and lipids of the membrane are vertical interactions that when disrupted cause a reduced density of spectrin and uncoupling of the lipid bilayer. This causes selective loss of the lipid bilayer and formation of spherocytes. Skeletal lattice proteins interact horizontally to form the stress-supporting skeletal protein lattice. Defects in these proteins result in mechanical instability and fragmentation of the cell. Inherited defects in membrane proteins produce the hereditary hemolytic anemias including hereditary spherocytosis, hereditary elliptocytosis, and hereditary pyropoikilocytosis. Abnormally permeable membranes can cause hereditary overhydrated and dehydrated stomatocytosis, but the specific abnormality of the membrane in these two disorders has not been definitively identified. Abnormalities in the lipid portion of the membrane can be inherited or acquired. These disorders result in the formation of acanthocytes.

Paroxysmal nocturnal hemoglobinuria (PNH), a stem cell disorder, is an acquired membrane abnormality in which complement-mediated destruction of the cell occurs. The defect is due to a lack of decay-accelerating factor (DAF) and membrane inhibitor of reactive lysis (MIRL) on the erythrocytes, secondary to the absence of a membrane glycolipid, glycosylphosphatidyl inositol (GPI) to anchor these proteins to the cell membrane. Hemolysis is intravascular, resulting in hemoglobinuria and decreased haptoglobin. Leukopenia and thrombocytopenia are common because these cells also are susceptible to complement destruction. The sucrose hemolysis test is a screening test for PNH. Although the Ham test was used in the past, immunophenotyping and the FLAER test are now the standard technologies for confirming a diagnosis of PNH.

Review Questions

Level I

1. What is the most prevalent erythrocyte morphology observed in hereditary spherocytosis? (Objectives 1, 3)

   A. small, spherical erythrocytes with little or no central pallor

   B. spheroidal erythrocytes with sharp irregular projections

   C. fragmented erythrocytes

   D. oval-shaped erythrocytes

2. What erythrocyte membrane disorder has erythrocytes that are thermally unstable and fragment when heated to 45–46°C? (Objectives 1, 3)

   A. hereditary spherocytosis

   B. hereditary elliptocytosis

   C. hereditary pyropoikilocytosis

   D. PNH

3. Choose the principal confirmation test for a diagnosis of hereditary spherocytosis. (Objective 3)
   A. autohemolysis test
   B. sucrose hemolysis test
   C. thermal stability test
   D. osmotic fragility test
4. Erythrocyte membrane disorders associated with known skeletal protein mutations include all of the following *except*: (Objective 1)
   A. hereditary spherocytosis
   B. hereditary overhydrated stomatocytosis
   C. hereditary elliptocytosis
   D. hereditary pyropoikilocytosis
5. Which of the following erythrocyte disorders is associated with abnormal membrane permeability? (Objective 2)
   A. hereditary elliptocytosis
   B. hereditary dehydrated stomatocytosis
   C. PNH
   D. HPP
6. Laboratory features associated with hereditary spherocytosis include: (Objective 3)
   A. spherocytes on the peripheral smear
   B. MCHC more than 36%
   C. increased osmotic fragility
   D. all of the above
7. The red blood cells in paroxysmal nocturnal hemoglobinuria (PNH) demonstrate a(n) _____ osmotic fragility test. (Objectives 4, 5)
   A. decreased
   B. increased
   C. normal
   D. unpredictable
8. Which of the following statements describes the basic principle behind the sucrose hemolysis test? This test determines whether: (Objective 6)
   A. a patient's erythrocytes incubated in their own serum will demonstrate increased hemolysis.
   B. a patient's erythrocytes are sensitive to complement lysis when exposed to acidified serum.
   C. the patient's erythrocytes incubated in a low ionic medium such as sucrose will demonstrate increased hemolysis.
   D. the patient's erythrocytes will resist acid elution.
9. The laboratory features associated with asymptomatic common hereditary elliptocytosis include: (Objective 3)
   A. fragmented erythrocytes on the peripheral smear
   B. increased osmotic fragility
   C. positive autohemolysis test
   D. mild reticulocytosis
10. The osmotic fragility test determines whether a patient's erythrocytes are osmotically fragile by measuring the amount of hemolysis that occurs: (Objective 4)
    A. after a patient's erythrocytes have been incubated for 24 hours in acidified serum
    B. when a patient's erythrocytes are incubated in various concentrations of hypotonic saline
    C. when the patient's erythrocytes are incubated in a sucrose solution
    D. hemolysis when the patient's erythrocytes are incubated in their own serum for 48 hours

Level II

1. Which of the following is the red blood cell membrane protein defect associated with hereditary pyropoikilocytosis? (Objective 1)
   A. deficiency of band 3
   B. defective ankyrin protein
   C. mutant spectrin
   D. excess cholesterol
2. Which of the following erythrocyte disorders will demonstrate an increased osmotic fragility pattern? (Objective 2)
   A. hereditary elliptocytosis and paroxysmal nocturnal hemoglobinuria
   B. hereditary overhydrated stomatocytosis and hereditary spherocytosis
   C. paroxysmal nocturnal hemoglobinuria and hereditary xerocytosis
   D. sickle cell anemia and thalassemia

Use this case study to answer questions 3 and 4.

A 5-year-old white male was admitted with the diagnosis of a fractured tibia following a playground accident. His admission laboratory results follow:

WBC	125×10^9/L	**Differential**	
RBC	3.6×10^{12}/L	Segmented	
Hb	10.2 g/dL	neutrophils	70%
Hct	27%	Lymphocytes	22%
MCV	96.3 fL	Monocytes	5%
MCH	28.3 fL	Eosinophils	2%
MCHC	38 g/dL	Basophils	1%

RBC morphology: Slight polychromasia and spherocytes present

Osmotic fragility test: Initial hemolysis: 0.65% NaCl
Complete hemolysis: 0.45% NaCl

3. Which erythrocyte index differentiates this membrane disorder from most of the other erythrocyte membrane disorders discussed in this chapter? (Objectives 2, 5)

   A. MCV

   B. MCH

   C. MCHC

   D. both the MCV and MCH

4. The patient's osmotic fragility test demonstrates that his erythrocytes have what type of osmotic fragility? (Objectives 2, 5)

   A. increased

   B. decreased

   C. normal

   D. questionable

5. Immunophenotyping for a diagnosis of PNH uses the following monoclonal antibodies: (Objective 4)

   A. CD55 and CD59

   B. CD11b/CD18

   C. CD33 and CD34

   D. CD56 and CD10

6. The RBC membrane permeability in hereditary overhydrated stomatocytosis is _____, and the cells have a(n) _____ osmotic fragility. (Objective 2)

   A. increased; decreased

   B. decreased; increased

   C. increased; increased

   D. decreased; decreased

7. The following results were obtained on an osmotic fragility test.

	Normal Control	Patient
Initial hemolysis	0.50%	0.65% NaCl
Complete hemolysis	0.30%	0.45% NaCl

   These results most closely relate to which of the following statements? (Objective 2)

   A. The patient's peripheral smear will reveal spherocytes.

   B. The patient's peripheral smear will reveal target cells.

   C. The patient's peripheral smear will reveal sickle cells.

   D. The test is out of control and should be repeated.

8. A decrease in the level of which erythrocyte enzyme occurs in PNH? (Objective 3)

   A. leukocyte alkaline phosphatase (LAP)

   B. acid phosphatase

   C. C5 convertase

   D. acetylcholinesterase

9. Which of the following statements best describes the role of the decay-accelerating factor (DAF)? (Objective 3)

   A. This regulatory protein enhances the amplification of complement lysis.

   B. This complement regulatory protein stimulates erythrocyte lysis.

   C. This regulatory protein prevents the amplification of C3/C5 convertase activity.

   D. None of these adequately describes the DAF role.

10. The function of the membrane inhibitor of reactive lysis (MIRL) is to: (Objective 3)

    A. induce erythrocyte aggregation

    B. interfere with the end stages of complement activation

    C. prevent production of an autoantibody

    D. all of the above

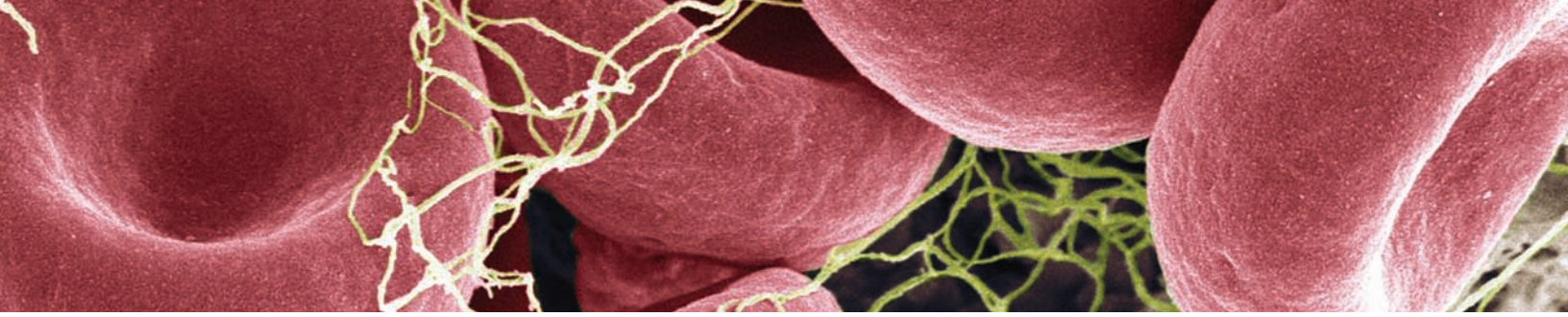
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Delauney J. The molecular basis of hereditary red cell membrane disorders. *Blood Rev*. 2007;21:1–20.
2. Gallagher PG, Jarolim P. Red blood cell membrane disorders. In: R Hoffman, EJ Benz Jr, SJ Shattil et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:623–43.
3. Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. *Lancet*. 2008;372:1411–26.
4. Mohandas N, Gallagher PG. Red cell membrane: past, present and future. *Blood*. 2008;112:3939–48.
5. Gallagher PG. Hematologically important mutations: Ankyrin variants in hereditary spherocytosis. *Blood Cells Mol Dis*. 2005;3:345–47.
6. van denAkker E, Satchwell T, Pellegrin S et al. Investigating the key membrane protein changes during the in vitro erythropoiesis of protein 4.2(-) cells (mutations Chartres 1 and 2). *Haematologica*. 2010;95:1278–86.
7. Xiuli A, Mohandas N. Disorders of red cell membrane. *Br J Haematol*. 2008;141:367–75.
8. Vives Corrons JL, Besson I. Red cell membrane Na^+ transport systems in hereditary spherocytosis: relevance to understanding the increased Na^+ permeability. *Ann Hematol*. 2001;80:535-539..
9. Gallagher PG. The red blood cell membrane and its disorders: hereditary spherocytosis, elliptocytosis and related diseases. In: Lichtman MA, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT. Eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill, Inc. 2010; 617–46.
10. Eber S, Lux SE. Hereditary spherocytosis—defects in proteins that connect the membrane skeleton to the lipid bilayer. *Semin Hematol*. 2004;41:118–41.
11. Brugnara C, Mohandas N. Red cell indices in classification and treatment of anemias: from M.M. Wintrobes's original 1934 classification to the third millennium. *Curr Opin Hematol*. 2013;20:222–30..
12. Crisp RL, Solari L, Vota D et al. A prospective study to assess the predictive value for hereditary spherocytosis using five laboratory tests (cryhemolysis test, eosin 5′-maleimide flow cytometry, osmotic fragility test, autohemolysis test and SDS-PAGE) on 50 hereditary spherocytosis families in Argentina. *Ann Hematol*. 2011;90:625–34.
13. D'Alcamo E, Agrigento V, Sclafani S et al. Reliability of EMA binding test in the diagnosis and management of hereditary spherocytosis in Italian patients. *Acta Haematol*. 2011;125:136–40.
14. Bolton-Maggs PHB, Langer JC, Iolascon A et al. Guidelines for the diagnosis and management of hereditary spherocytosis–2011 update. *Br J Haematol*. 2011;156: 37–49.
15. Buesing KL, Tracy ET, Kiernan C et al. Partial splenectomy for hereditary spherocytosis: a multi-institutional review. *J Pediatr Surg*. 2011;46:178–83.
16. Gallagher PG. Update on the clinical spectrum and genetics of red blood cells membrane disorders. *Curr Hematol Rep*. 2004;3:85–91.
17. Gallagher PG. Hereditary elliptocytosis: spectrin and protein 4.1R. *Semin Hematol*. 2004;41:142–64.
18. King MJ, Zanella A. Hereditary red cell membrane disorders and laboratory diagnostic testing. *Int J Lab Hem*. 2013;35:237-43.
19. Ficke B, Argent AC, Chetty MC et al. The "stomatin" gene and protein in overhydrated hereditary stomatocytosis. *Blood*. 2003;102:2268–77.
20. Bruce LJ. Hereditary stomatocytosis and cation leaky red cell—recent developments. *Blood Cells Mol Dis*. 2009;42:216–22.
21. Van Kim CL, Colin Y, Carton JP. Rh proteins: key structural and functional components of the red cell membrane. *Blood Rev*. 2006;20:93–110.
22. Gallagher PG. Red cell membrane disorders. *Hematol Am Soc Hematol Edu*. 2005;1:13–18.
23. Vassiliadis T, Mpoumponaris A, Vakalopoulous S et al. Spur cells and spur cell anemia in hospitalized patients with advanced liver disease: incidence and correlation with disease severity and survival. *Hepatol Res*. 2010;40:161–71.
24. Hooper AJ, vanBockxmeer FM, Burnett JR. Monogenic hypocholesterolaemic lipid disorders and apolipoprotein B metabolism. *Crit Rev Clin Lab Sci*. 2005;42:515–45.
25. Wong P. A basis of the acanthocytosis in inherited and acquired disorders. *Med Hypotheses*. 2004;62:966–69.
26. Nishiwaki N, Ikewaki K, Bader G et al. Human lecithin cholesterol acylteransferase deficiency in vivo kinetics of low-density lipoprotein and lipoprotein X. *Arterioscler Thromb Vasc Biol*. 2006;26:1370–75.
27. Parker C, Ormine M, Richard S et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. *Blood*. 2005;106:3699–709.
28. Brodsky RA. Paroxysmal nocturnal hemoglobinuria. In: Hoffman R, EJ Benz Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Churchill Livingston; 2009; 385–94.
29. Borowitz MJ, Craig FE, DiGiuseppe JA et al. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. *Cytometry B Clin Cytom*. 2010;188:211–30.
30. Hillmen P, Young NS, Schubert J et al. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2006;255:1233–43.30.



18 Hemolytic Anemia: Enzyme Deficiencies

MARTHA LAKE, EDD
DAN BESSMER, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Identify the two main pathways by which erythrocytes catabolize glucose.
2. Explain the role of erythrocyte enzymes in maintaining the cell's integrity, and describe how deficiencies in these enzymes can lead to anemia.
3. Identify the most common erythrocyte enzyme deficiency.
4. Describe the inheritance pattern for glucose-6-phosphate dehydrogenase (G6PD).
5. Explain how the diagnosis of G6PD deficiency is made.
6. List the tests used to detect G6PD deficiency and describe their principles.
7. Recognize the erythrocyte morphology in a Romanowsky-stained blood smear associated with G6PD deficiency.
8. Identify common compounds that induce anemia in G6PD deficiency.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Recommend appropriate laboratory testing and interpret results for suspected G6PD deficiency following a hemolytic episode.
2. Explain the function of glutathione in maintaining cellular integrity.
3. Associate the mechanisms of hemolysis with defects in the glycolytic and hexose monophosphate shunt pathways.
4. Correlate clinical and laboratory findings with the common G6PD variants.
5. Diagram the reaction catalyzed by pyruvate kinase, and explain how a defect of this enzyme can cause hemolysis.
6. Recognize erythrocyte morphology associated with pyruvate kinase deficiency.
7. Review and interpret laboratory findings in a case study presentation of G6PD deficiency.

Chapter Outline

Key Terms

Bite cell
Blister cell
Chronic nonspherocytic hemolytic anemia
Favism
Hypoxia
Lyonization

Background Basics

The information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review the following concepts from previous chapters and other resources:

Level I and Level II

- Describe the normal erythrocyte metabolic processes. (Chapter 5)
- Recognize RBC morphology as it relates to hemolytic disease processes. (Chapters 10, 11)
- Understand basic enzymology and techniques to measure enzyme activity. (Other resources)

CASE STUDY

We will address this case study throughout the chapter.

The patient, Henry, is a 20-year-old African American male who was in the process of being assigned to West Africa for a 12-month period with the Peace Corps. Because of the high prevalence of malaria in the area, he was started on antimalarial prophylaxis (primaquine) 3 days prior to his flight to Africa. Twenty-four hours after starting the medication, he developed fever, chills, and general malaise. He subsequently reported to the emergency department and was admitted to the hospital for observation and additional testing.

The physical exam revealed a normal appearing, well-nourished adult male in no acute distress. His family history was noncontributory, and he had no known drug allergies. Laboratory analysis yielded the following:

CBC	
WBC	12×10^9/L
Hb	9.1 g/dL (91 g/L)
Hct	27% (0.27 L/L)
MCV	90 fL
PLT	423×10^9/L
Total bilirubin	5.0 mg/dL
Conjugated bilirubin	0.2 mg/dL
Unconjugated bilirubin	4.8 mg/dL
Haptoglobin	39 mg/dL

Based on the clinical history and these laboratory results, consider what could have precipitated this patient's condition.

OVERVIEW

Defects within the erythrocyte can shorten the red cell life span, resulting in hemolytic anemia. These defects are intrinsic to the cell and can involve the erythrocyte membrane (Chapter 17), hemoglobin (Chapter 13), or enzymes. The two most common enzyme defects within the erythrocyte are glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK). Of these two enzyme deficiencies, G6PD defects are found more frequently and affect the hexose monophosphate shunt. PK defects are the second most common enzyme deficiency and affect the glycolytic pathway. Thus, the chapter begins with a description of the role of these two pathways in erythrocyte metabolism and a general overview of the clinical and laboratory findings in enzyme deficiencies associated with these pathways. G6PD and PK enzyme deficiencies are discussed in detail in the format of pathophysiology, clinical and laboratory findings, and therapy.

INTRODUCTION

Even though the mature erythrocyte lacks a nucleus, mitochondria and ribosomes, the cell is still metabolically active (Chapter 5). Metabolism provides energy to keep hemoglobin iron in the reduced state, pump ions across the cell membrane, keep the sulfhydryl groups in enzymes and other proteins in the reduced state, and maintain the cell's shape. These activities are essential for red cell survival. The erythrocyte life span can be significantly shortened if the cell is intrinsically abnormal. Lacking the ability to synthesize proteins and undergo oxidative phosphorylation for ATP production, the mature erythrocyte depends entirely on anaerobic metabolism for its energy needs. Maturation of the reticulocyte is associated with a decline in the activity of some enzymes, but the activity usually is relatively stable in the mature erythrocyte.[1] Because the erythrocyte cannot synthesize new enzymes, the amount of enzyme present for normal cell function is limited.

An inherited deficiency in one of the erythrocyte enzymes can disrupt cellular metabolism and compromise the integrity of the cell membrane or hemoglobin and cause hemolysis. Refer to Table 18-1 ★ for the more common hereditary enzyme deficiencies known to cause hemolytic anemia. Most are associated with two erythrocyte metabolic pathways: the hexose monophosphate (HMP) shunt and the glycolytic (Embden-Meyerhof, EM) pathway. To understand how defects in these two pathways can result in hemolysis, they will be reviewed. (For a more thorough discussion of each, review Chapter 5.)

★ **TABLE 18-1** Erythrocyte Enzyme Deficiencies Associated with Congenital (Chronic) Nonspherocytic Hemolytic Anemia

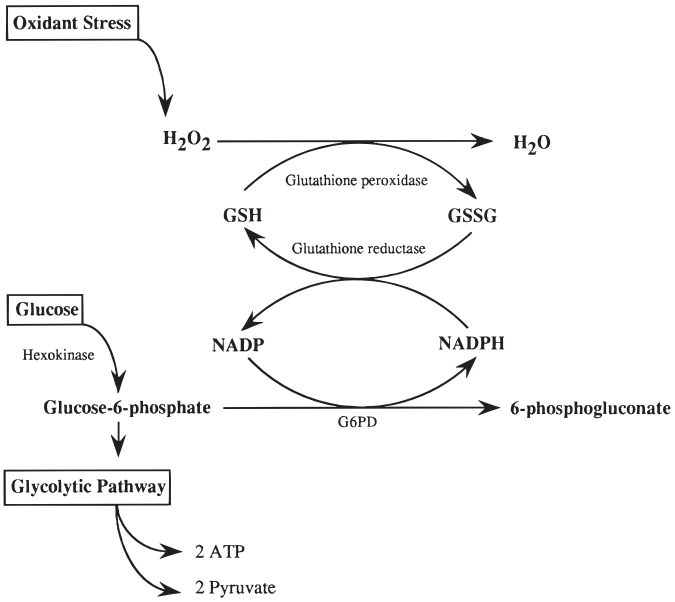
Metabolic Pathway	Enzyme Deficiency
Glycolytic (Embden-Meyerhof)	Pyruvate kinase (PK)
	Glucose phosphate isomerase (GPI)
	Hexokinase (HK)
	Phosphoglycerokinase (PGK)
	Phosphofructokinase (PFK)
	Triosephosphate isomerase (TPI)
Hexose monophosphate	Glucose-6-phosphate dehydrogenase (G6PD)
	Glutathione synthetase
	Glutamylcysteine synthetase
	Glutathione reductase
Nucleotide	Pyrimidine 5′-nucleotidase (P5N)

Hexose Monophosphate Shunt

Glucose, the cell's primary metabolic substrate, enters the cell in a carrier-mediated, energy-free transport process and is catabolized via the glycolytic pathway or the HMP shunt. The HMP shunt catabolizes approximately 10% of the glucose, and is essential for maintaining adequate concentrations of reduced glutathione (GSH). GSH protects the erythrocyte from oxidant damage: it maintains hemoglobin in the reduced functional state and safeguards vital cellular enzymes from oxidation. GSH is maintained at high levels by conversion of NADPH to NADP; NADP is reduced back to NADPH in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PD) (Figure 18-1 ■). When the cell is exposed to an oxidizing agent, more NADPH is consumed as part of the cell's protective mechanism to maintain sufficient levels of GSH, and G6PD is required to restore levels of NADPH. Oxidized GSH (GSSG) is also actively transported out of the erythrocyte. If enzymes in the HMP shunt are deficient, the cell's reducing power is compromised, and oxidized hemoglobin accumulates, subsequently denaturing in the form of Heinz bodies. Heinz bodies aggregate at the cell membrane, causing membrane damage. As the cells pass through the spleen, the macrophages attempt to remove the Heinz bodies, leading to premature extravascular hemolysis. The most common enzyme deficiency of the HMP shunt is G6PD deficiency.

Glycolytic Pathway

Most of the cell's energy is produced via glycolysis in the glycolytic pathway. About 90% of the glucose is metabolized by this pathway as one mole of glucose is catabolized to lactic acid with a net production of two moles of ATP. ATP is needed for active cation transport of Na^+, K^+, and Ca^{++}, maintaining membrane deformability and the normal erythrocyte biconcave disc shape.[1] The erythrocyte's ability to deform is an important determinant of its survival. Deficiencies in enzymes of the glycolytic pathway decrease ATP production and lead to hemolysis. The mechanism of hemolysis is related to decreased ATP, leading to impaired cation pumping. Membrane integrity is compromised and increased osmotic fragility results. The osmotically fragile cells are trapped in the hostile splenic environment and phagocytized.



■ **FIGURE 18-1** G6PD is needed for maintaining adequate quantities of glutathione (GSH), an important buffer to oxidants within the erythrocyte. As GSH reduces H_2O_2 to H_2O, it is oxidized (GSSG). G6PD generates NADPH in the conversion of glucose-6-phosphate to 6-phosphogluconate. NADPH, in turn, regenerates reduced glutathione from oxidized glutathione.

Heinz bodies are not formed because the cell's reducing power is primarily linked to the HMP shunt, which is not affected.

The Rapoport-Luebering shunt of the glycolytic pathway provides the erythrocyte with 2,3-bisphosphoglycerate (2,3-BPG). When 1,3-BPG is shunted to 2,3-BPG instead of directly to 3-PG, a critical reaction producing ATP is bypassed and there is no net gain of ATP from glycolysis. The activity of this shunt is stimulated during **hypoxia** to facilitate oxygen delivery to the tissues. 2,3-BPG binds to hemoglobin, decreasing hemoglobin's oxygen affinity and making more oxygen available to the tissues.

Clinical and Laboratory Findings in Erythrocyte Enzyme Deficiencies

Most erythrocyte enzyme deficiencies are inherited as autosomal recessive traits. However, the most common enzyme deficiency, G6PD, is inherited as an X-linked recessive (sex-linked) disorder. Individuals homozygous for the autosomal recessive enzyme deficiencies and males with X-linked G6PD deficiency are most likely to be symptomatic. The clinical presentation is variable, depending on the specific mutation inherited and can range from no hemolysis/anemia, to intermittent hemolysis, to a chronic hemolytic state. Affected individuals generally have a normocytic, normochromic anemia, reticulocytosis, hyperbilirubinemia, and neonatal jaundice. The direct antiglobulin test (DAT) is negative, indicating an absence of antibodies coating the erythrocytes, and there is no evidence suggesting a defect in either the erythrocyte membrane or hemoglobin.

The anemias associated with inherited defects of erythrocyte metabolism can be acute or chronic, depending on the mutant

enzyme inherited. Some mutations cause low enzyme activity or instability, whereas other mutations are associated with enzymes that have moderately to mildly decreased activity. Some mild types are associated with hemolysis only under stressful conditions such as administration of oxidant drugs, whereas severely deficient or unstable types cause chronic hemolysis in the absence of stress. The chronic hemolytic types are often collectively referred to as *hereditary nonspherocytic hemolytic anemia*. Crosby introduced this term in 1950 to describe hemolytic anemia that appeared at an early age but in whom testing revealed a normal osmotic fragility,[1] and it was used to differentiate a hereditary hemolytic anemia that was not hereditary spherocytosis. This heterogeneous group of anemias does not have significant poikilocytosis. Although the anemias might not be life threatening, they can be disabling and lead to debilitating complications. Although anemias other than those caused by enzyme mutations can also be chronic, hemolytic, and nonspherocytic, they are not included in this group (e.g., sickle cell disease, thalassemia).

Diagnosis

Definitive diagnosis of enzyme deficiencies requires spectrophotometric measurement of the suspected deficient enzyme and/or molecular testing for the specific gene. The timing of enzymatic testing and interpretation of the results is important for accurate diagnosis. With some enzyme deficiencies, enzyme activity is nearly normal in reticulocytes but decreases as the cell ages (e.g., G6PD-A$^-$). Thus, depending on the age distribution of the circulating cells and the degree of reticulocytosis, the enzyme content can appear normal. For this reason, the patient's blood should not be collected for testing immediately after a hemolytic episode when most of the enzyme-deficient cells have been hemolyzed and there is a compensatory reticulocytosis. Diagnosis during or shortly after a hemolytic episode can be made if the blood is centrifuged and the older dense erythrocytes at the bottom of the column of blood are tested.[2,3] If a patient has been recently transfused, testing should be delayed until the transfused cells are no longer present.[1]

☑ CHECKPOINT 18-1

Transfusion of red blood cells in a patient with chronic nonspherocytic, hemolytic anemia as the result of an erythrocyte enzyme deficiency does not reverse or prevent the recipient's condition. However, a transfusion does help to raise the patient's hemoglobin. If tests are performed to quantitate the enzyme after a transfusion, the results can be within the normal reference intervals. Explain.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

G6PD deficiency is the most common erythrocyte enzyme disorder. It was first recognized during the Korean War when 10% of African American soldiers who were given the antimalarial drug primaquine developed a self-limited hemolytic anemia. Currently, the U.S. Army, Navy, and Air Force screen all soldiers, civilians, and other beneficiaries for G6PD and prohibit prescriptions for primaquine in G6PD-deficient individuals.[4] G6PD deficiency is found worldwide but occurs most frequently in people from the Mediterranean area, Africa, and China.

The geographic distribution coincides with that of malaria, suggesting that G6PD deficiency may provide protection against this disease. One mechanism for this protection could be oxidant injury to the parasite. Due to the cell's inability to restore NADPH and GSH, the parasite may be more vulnerable to the reactive intermediates formed when the parasite breaks down hemoglobin.[5] Evidence also suggests that G6PD-deficient erythrocytes containing earlier stages of parasite maturation (ring stage) may be more readily phagocytized.[6,7] The exact mechanism of this hypothesized protection is unknown, however, and epidemiological and clinical studies have not consistently shown a clear connection between protection against malaria and G6PD deficiency.[8,9,10] Nevertheless, the geographic coincidence is compelling.

Because G6PD deficiency is a sex-linked disorder (the cytogenetic location of the gene, *G6PD*, is Xq28), it is fully expressed in males with a genetic mutation and in females homozygous or double heterozygous for a mutant allele. More than 400 G6PD variants have been identified.[1]

G6PD deficiency is heterogeneous with differences in severity among races, sexes, and the mutant variant inherited. The majority of people with a G6PD variant allele have no clinical expression of the deficiency unless they are exposed to oxidative chemicals or drugs, or have severe infections. They also can have neonatal jaundice. See Table 18-2 ★ for compounds that have been associated with hemolytic anemia in G6PD deficiency.

☑ CHECKPOINT 18-2

Oxidant compounds are harmful because they result in the production of toxic peroxides or other oxygen radicals that overwhelm the body's natural mechanisms to scavenge them. Why is the protection against oxidants easily compromised in G6PD deficiency?

Pathophysiology

G6PD is necessary for maintaining adequate levels of GSH for reducing cellular oxidants (Figure 18-1). In G6PD deficiency, the generation of NADPH, and subsequently GSH, is impaired and cellular oxidants accumulate. The buildup of cellular oxidants leads to erythrocyte injury and both intravascular and extravascular hemolysis. Oxidants cause the oxidation of free –SH groups in hemoglobin and other intracellular proteins, forming disulfide bridges. Oxidized hemoglobin has decreased solubility and precipitates as Heinz bodies. Heinz bodies attach to the erythrocyte membrane, causing increased membrane permeability to cations, osmotic fragility, and cell rigidity. Heinz bodies are removed from the erythrocytes by splenic macrophages, producing bite cells and blister cells. With progressive membrane loss, spherocytes can be formed (Figure 18-2 ■). Spherocytes are less deformable than normal cells and become trapped and hemolyzed in the spleen (extravascular hemolysis). Heinz bodies require a supravital stain to

★ **TABLE 18-2** Compounds Associated with Hemolysis in G6PD Deficiency

Antimalarials	Primaquine
	Pamaquine
	Pentaquine
	Quinacrine (Atabrine)
Sulfonamides	Sulfanilamide
	Sulfacetamide
	Sulfapyridine
	Sulfamethoxazole (Gantonol)
	Sulfasalazine (Azulfidine)
Sulfones	Thiazosulfone
	Diaphenylsulfone (DDS, Dapsone)
	Sulfoxone (Diasone)
Nitrofurans	Nitrofurantoin (Furadantin)
Analgesics	Acetanilid
Miscellaneous	Fava beans
	Hydroxylamines
	Methylene blue
	Naphthalene (moth balls)
	Naxidlic acid
	Niridazole
	Phenylhydrazine
	Toluidine blue
	Trinitrotoluene (TNT)

be visualized because they are not evident on Romanowsky-stained blood smears. Oxidant stress also can oxidize membrane lipids and proteins. This disruption of the membrane structural integrity results in removal of the cell from the circulation by splenic macrophages. Cell membrane damage can occasionally be severe enough for the cell to hemolyze in the circulation. This intravascular hemolysis can be acute and accompanied by hemoglobinemia and hemoglobinuria.

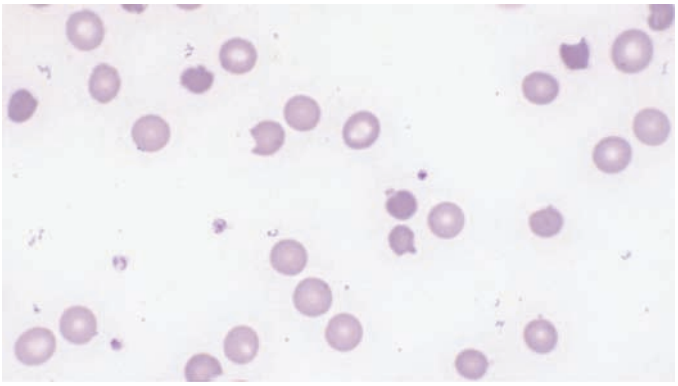


FIGURE 18-2 Peripheral blood from a patient with G6PD deficiency during a hemolytic episode. There are erythrocytes with a portion of the cell, known as a *bite cell*, missing. The spleen pits the Heinz bodies with a portion of the cell producing these misshapen erythrocytes. Some of the cells reseal and become spherocytes (Wright-Giemsa stain, 1000× magnification).

G6PD activity is highest in young cells and decreases rapidly as cells age. Reticulocytes have approximately 5 times higher enzyme activity than the oldest circulating erythrocytes. However normal erythrocytes use only 0.1% of their maximum G6PD enzyme capacity.[11] Thus, under normal conditions, even older cells retain enough G6PD activity to maintain adequate GSH levels. This explains why most G6PD-variant cells can maintain normal function and hemolysis is sporadic. When erythrocytes are overwhelmed by excessive oxidant stress, the G6PD activity becomes inadequate to maintain normal metabolic function in individuals who have inherited a G6PD variant. Those cells that are most deficient (i.e., the oldest) undergo oxidative damage and are rapidly removed from circulation. With most G6PD variants, hemolysis is self-limited (e.g., hemolysis stops after a time even if the oxidant stress continues). Self-limited hemolysis occurs because the older, most G6PD-deficient erythrocytes initially are destroyed, but the younger cells remain because they have sufficient enzyme activity to avoid hemolysis. The reticulocytes released from the bone marrow in response to the hemolytic episode also have enough enzyme activity to maintain metabolic activity even under oxidant stress. However, it is important to recognize that under the stress of severe oxidants (drugs, chemicals), even normal cells can experience oxidant damage and hemolysis.

☑ CHECKPOINT 18-3

Erythrocyte morphology should always be examined carefully. The ability to pick up subtle clues regarding the cause of a disease process can be acquired from a comprehensive evaluation of abnormal erythrocyte morphology. How is this likely to aid the diagnosis of G6PD deficiency?

G6PD Variants

More than 400 variants of the G6PD enzyme have been identified.[1] Many of them differ in activity, stability, and/or electrophoretic mobility. The World Health Organization (WHO) has categorized the variant enzymes into five classes according to the degree of deficiency and hemolysis[12] (Table 18-3 ★). Most of the identified variants actually have normal enzyme activity. Deficient enzyme variants tend to have mutations clustered in domains responsible for stable dimerization of the active form of the protein, or the NADP binding site.[13] Except for G6PD-B, G6PD-A^+, and G6PD-A^-, variants are given geographic or other types of names.

Females with G6PD Deficiency

Female heterozygotes for G6PD deficiency always contain two populations of cells, one normal and one G6PD deficient. In contrast, all cells in affected males are G6PD deficient. The dual population in females is caused by **lyonization**, the random inactivation of one X chromosome in each hematopoietic stem cell. Depending on the proportion of abnormal erythrocytes and the nature of the inherited variant, females might have no clinical expression of the deficiency or be affected as severely as males. Although rare, there are case reports of

★ **TABLE 18-3** WHO Classification of Mutant G6PD Alleles

Class	G6PD Activity	Hemolysis	Important Variants
I	Severely deficient	Chronic nonspherocytic hemolytic anemia, not self-limited	Minnesota, Iowa
II	Severely deficient (<10%)	Acute, episodic, can be chronic and not self-limited	G6PD-Mediterranean, common severe oriental variants
III	Moderately to mildly deficient (10–60%)	Acute, episodic	G6PD-A$^-$, G6PD-Canton
IV	Normal (60–150%)	Absent	G6PD-B, G6PD-A$^+$
V	Increased activity	Absent	

homozygous or double heterozygous-deficient females.[14] Web Figure 18-1 illustrates the expected progeny from G6PD-deficient males or females.

Clinical Findings

A spectrum of clinical presentations is associated with this disorder: (1) acute, acquired hemolytic anemia (episodic), including favism; (2) hereditary (congenital) nonspherocytic hemolytic anemia (chronic); and (3) neonatal hyperbilirubinemia with jaundice. Homozygotes and heterozygotes can be symptomatic, depending on the severity of the deficiency. G6PD deficiency is most common in those of African, Asian, Mediterranean, and Middle Eastern heritage.

Acute, Acquired Hemolytic Anemia

Most persons with G6PD deficiency have no clinical symptoms, and they are not anemic. Diagnosis usually occurs during or after infectious illnesses or following exposure to certain drugs because these conditions commonly precipitate hemolytic episodes. Hemolysis is variable and depends on the degree of oxidant stress, the G6PD variant, and sex of the patient. The symptoms are those of an acute intravascular hemolytic anemia (Chapter 11). Drug-induced hemolysis usually occurs within 1 to 3 days after exposure to the drug. Sudden anemia develops with a 3–4 g/dL drop in hemoglobin. Jaundice is not prominent. The patient can experience abdominal and low back pain, as well as dark or black urine due to hemoglobinuria. In one study of 35 G6PD-deficient children in India, the most common significant complication, occurring in >50% of the cases, was renal failure.[15] Hemoglobinemia is prominent. Often, however, hemolysis is less striking and is not accompanied by hemoglobinuria or conspicuous symptoms.

Favism refers to the sudden severe hemolytic episode that develops in some individuals with G6PD deficiency after the ingestion of fava beans (broad beans). Hemolytic episodes occur in much the same way as drug-induced hemolytic episodes. The most likely components of the bean responsible for the sensitivity are isouramil and divicine.[16] These acute hemolytic episodes were thought to be associated with severe G6PD deficiency, especially the G6PD-Mediterranean variant. It is now known that other forms of G6PD deficiency are also associated with favism, including G6PD-A$^-$ and G6PD-Aures, a variant identified in Algerian subjects.[17]

The hemolysis associated with favism is similar to the acute hemolytic episodes that occur after primaquine administration in individuals with the G6PD-A$^-$ variant. Consumption of fava beans is widespread in the Mediterranean area and the Middle East. The first signs of favism are malaise, severe lethargy, nausea, vomiting, abdominal pain, chills, tremor, and fever.[1] Hemoglobinuria occurs a few hours after ingestion of the beans. Persistent hemoglobinuria usually prompts the individual to seek medical attention. Jaundice can be intense. Severe favism usually affects children between the ages of 2 and 5 years. The incidence in young children is changing in some countries, however, due to neonatal screening and parental education. Even though favism occurs more often in males due to the X-linked nature of the disease, heterozygous females can also be affected depending on the proportion of enzyme-deficient erythrocytes present.

Hereditary Nonspherocytic Hemolytic Anemia

Hereditary nonspherocytic hemolytic anemia syndrome is associated with G6PD variants (WHO class I) that have low in vitro activity or are markedly unstable. Hemolysis is chronic and not associated with ingestion of drugs or infections, although drugs and infections can exacerbate the hemolysis. The hemolysis is usually compensated so anemia can be mild. Reticulocytosis is in the range of 4–35%. This type of G6PD deficiency is often referred to as **chronic nonspherocytic hemolytic anemia**.

Leukocyte and platelet G6PD are controlled by the same gene locus as erythrocyte G6PD. Thus, some patients with G6PD-deficient red cells have been found also to have leukocyte G6PD deficiency. An increase in pyogenic infections has been reported in those with <5% normal activity.[5,18–20] However, a group of Israeli patients with severe G6PD deficiency without an increased incidence of infections had bactericidal activity in their G6PD-deficient neutrophils within the range of healthy controls.[21] Considerable fluctuation of enzyme activity in leukocytes was found to depend on the time of day it was measured. This fluctuation can produce enough NADPH to initiate the respiratory burst and prevent infection. Neutrophils also are relatively short-lived cells, so those with unstable variants might not show functional impairment.

Neonatal Hyperbilirubinemia

Some neonates with G6PD deficiency have severe hyperbilirubinemia with the potential of kernicterus if untreated. The prevalence of this severe jaundice occurs twice as often in G6PD-deficient male neonates compared with neonates from the general population. The mechanism is unknown. Although hemolysis can be present, other factors, including inability to conjugate and clear bilirubin, seem to play a more important role.[22] In one study, G6PD deficiency was identified

in at least 21% of infants who were readmitted with kernicterus.[23] Treatment can include phototherapy and exchange transfusion.

Healthy neonates and preterm infants (29–32 weeks' gestation) have higher G6PD activity than do adults. However, the higher activity does not affect the diagnosis of G6PD because neonates and preterm infants with G6PD deficiency have lower activity than do normal neonates.[24]

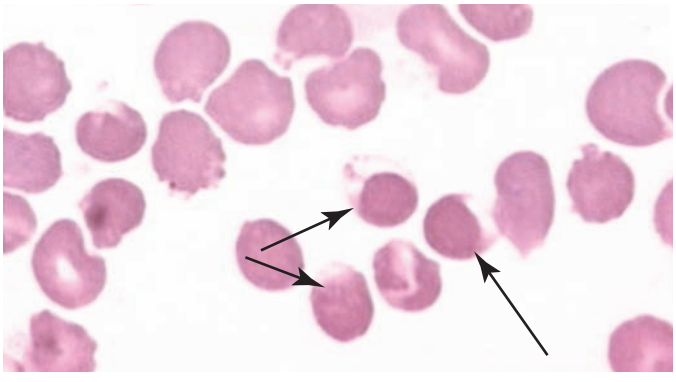
Laboratory Findings

Anemia is absent and peripheral blood findings are normal for most of the common G6PD variants associated with episodic disease except during hemolytic episodes. Patients with the hereditary nonspherocytic hemolytic anemia form can exhibit chronic hemolysis. During or immediately following a severe hemolytic episode, polychromasia, occasional spherocytes, small hypochromic cells, erythrocyte fragments, blister cells, and bite cells can be seen on the blood smear. **Bite cells** (degmacytes) have a chunk of the cell removed from one side and are thought to be formed when phagocytes in the spleen remove the denatured hemoglobin (Heinz bodies) bound to the cell membrane. They are frequently thought to be typical of G6PD deficiency (Figure 18-2). However, bite cells are more characteristic of drug-induced oxidant hemolysis in individuals with normal hemoglobin and enzyme activity.[25]

A peculiar cell, the **blister cell**, referred to by a variety of descriptive terms (irregularly contracted cell, eccentrocyte, erythrocyte hemighost, double-colored erythrocyte, and cross-bonded erythrocyte) has been described in G6PD deficiency after oxidant-related hemolysis (Figure 18-3 ■). The red cell membrane is oxidized, and the hemoglobin is confined to one side of the cell, whereas the other side is transparent. The transparent side often contains Heinz bodies and has flattened membranes in which the opposing membrane sides are juxtaposed. This cross-bonding of the membrane appears to decrease deformability and destine the cell for phagocytosis by macrophages. These cells have a decreased volume and increased MCHC.

A variety of abnormal laboratory findings related to hemolysis can be found during or after a hemolytic episode. Reticulocytosis is characteristic following a hemolytic episode.[26] Leukocytes can increase, but platelets are usually normal. Unconjugated bilirubin and serum lactate dehydrogenase (LD) can be increased. Haptoglobin commonly decreases during the acute hemolytic phase. Absence of haptoglobin in the recovery stage indicates chronic hemolysis.

Definitive diagnosis depends on the demonstration of a decrease in erythrocyte G6PD activity. In affected individuals, the enzyme activity can appear normal during and for a time after a hemolytic episode because older cells with less G6PD are preferentially destroyed and the newly released reticulocytes have higher activity. A reticulocytosis of >7% is generally associated with a normal enzyme screen after hemolysis.[27] For this reason, assays for G6PD should be performed 2 to 3 months after a hemolytic episode. In G6PD-Mediterranean, however, even young cells have gross deficiencies of G6PD, and enzyme activity appears abnormal even with reticulocytosis. Both severe and mild types of G6PD deficiency are detected by measuring the enzyme if the patient is not undergoing hemolysis.



■ **FIGURE 18-3** The arrows are pointing to blister cells, which have various names (see the chapter text). Hemoglobin is condensed to one side of the cell, leaving a transparent area (blister) on the other. These cells can be found in G6PD-deficient individuals after a hemolytic attack (Wright-Giemsa stain, 1000× magnification).

CASE STUDY (continued from page 335)

Because of the low hemoglobin, Henry was transfused with 2 units of packed red cells. Examination of the peripheral blood smear was remarkable for occasional spherocytes and bite cells. This blood smear finding, presentation of anemia, and onset of illness coinciding with the initiation of primaquine suggested shortened RBC life span due to oxidative damage.

1. What test should be considered after finding bite cells on a blood smear?

Qualitative Fluorescent Spot Test

The fluorescent spot test (Beutler fluorescent spot test) is a rapid, reliable, and sensitive screening test for G6PD deficiency. Whole blood is added to a mixture of glucose-6-phosphate (G6P), NADP, and saponin. A drop of this mixture is placed on a piece of filter paper and examined under ultraviolet light for fluorescence. The G6PD enzyme present in erythrocytes normally metabolizes G6P, producing NADPH, which fluoresces. NADP does not fluoresce, so lack of fluorescence indicates G6PD deficiency. Although this test often produces false negative results, especially after a hemolytic episode, it is useful in identifying severe deficiencies. A recent modification of the fluorescent spot test is to report "intermediate" fluorescence along with "absent" and "present" fluorescence. This modification is recommended to improve the diagnosis of G6PD deficiency in heterozygous females and individuals with moderate deficiencies.[28] In a recent clinical trial, the fluorescent spot test had 26% false negatives and 2% false positives.[29]

Dye Reduction Test

The dye reduction screening test incubates a hemolysate of patient's blood with G6P, NADP, and the dye brilliant cresyl blue. If the hemolysate contains G6PD, the NADP is reduced to NADPH, which in

turn reduces the blue dye to its colorless form. The time it takes for this change to occur is inversely proportional to the amount of G6PD present. Normal blood is also tested as a control. The test is specific and is available as a commercial kit. A recent study of a point-of-care dye reduction test kit reported a significant problem with false negatives.[30] Another commercial kit uses a lateral flow test strip and is read visually. This kit had a positive predictive value of 72% with heparinized blood and 65% with EDTA-preserved blood.[31] A quantitative version of this test measures the rate of reduction of NADP to NADPH at 340 nm in a spectrophotometer.

Ascorbate Cyanide Test

The ascorbate cyanide test is the most sensitive screening test for detecting heterozygotes and G6PD deficiency during hemolytic episodes. The test is not specific for G6PD deficiency but also detects other defects or deficiencies in the HMP shunt. The test is also positive in paroxysmal nocturnal hemoglobinuria (PNH), PK deficiency, and unstable hemoglobin disorders. The test's principle is that G6PD-deficient cells fail to reduce hydrogen peroxide. A patient's blood sample is incubated with sodium ascorbate, sodium cyanide, and glucose. Hydrogen peroxide is generated by the interaction of ascorbate with hemoglobin. The sodium cyanide inhibits the activity of normal erythrocyte catalase, which catalyzes the degradation of hydrogen peroxide. Erythrocytes deficient in G6PD cannot reduce the peroxide, and hemoglobin is oxidized to methemoglobin, which imparts a brown tint to the solution.

Cytochemical Staining

The enzyme activity in individual cells is detected by the cytochemical staining method. The G6PD in the red cells reacts with a sensitive tetrazolium salt to ultimately form formazan. The level of formazan is directly related to the G6PD activity. The activity in the cells can be scored by hand, but automated methods utilizing flow cytometry are available.[32]

Quantitation of G6PD

Screening tests for the enzyme are recommended before performing more expensive quantitative tests. It has been shown that semiquantitative tests that use a low cutoff of 2.10 U/g Hb fail to detect most heterozygous female neonates. Sensitivity of the test can be increased if the cutoff is raised to 2.55 U/g Hb for females and to 2.35 U/g Hb for males.[33] A blood sample can be submitted to a reference laboratory that can perform the quantitative erythrocyte enzyme assays. G6PD enzyme levels can be quantitated by incubating an erythrocyte hemolysate with G6P and NADP, and measuring the rate of reduction of NADP to NADPH at 340 nm in a spectrophotometer.

Molecular Methods

In the case of heterozygous females or when the quantitation of the enzyme can yield misleading results such as during or after a hemolytic episode, it is best to perform polymerase chain reaction (PCR) tests to reveal the genetic abnormality. In a DNA-based screening test, DNA is extracted from spots of dried blood and then submitted for PCR. Fluorescent-labeled probes are used to detect mutant G6PD alleles.

CASE STUDY *(continued from page 340)*

A spectrophotometric assay for G6PD was performed on Henry's peripheral blood. The result was borderline normal, and a preliminary diagnosis of G6PD deficiency was made. Primaquine usage was discontinued, and the patient recovered without complications. Upon follow-up, the patient was retested for G6PD deficiency and was found to be abnormal, confirming the diagnosis.

2. Why was the initial G6PD test result normal but the repeat test abnormal?

Differential Diagnosis

G6PD deficiency has similar clinical features and some overlapping laboratory test results that are similar to drug-induced hemolysis associated with unstable hemoglobins.[1] Abnormalities in hemoglobin electrophoresis and the hemoglobin stability test help identify the unstable hemoglobins but are normal in G6PD deficiency. The ascorbate cyanide test can be abnormal in both unstable hemoglobins and G6PD deficiency, but the G6PD assay and fluorescent screening test are positive only in G6PD deficiency. Exclusion of anemias associated with defects of the erythrocyte membrane can be made as these anemias have characteristic poikilocytosis (e.g., spherocytes, ovalocytes, stomatocytes).

Therapy

Most of the patients with G6PD deficiency are asymptomatic and do not experience chronic hemolysis; thus, no therapy is indicated. However, patients should avoid exposure to the oxidant drugs and foods that can precipitate hemolytic episodes. In acute hemolytic episodes, supportive therapy including blood transfusions, treatment of infections, and removal of the precipitating agent are used. Exchange transfusion may be necessary in cases of severe neonatal jaundice. Dialysis can be indicated in patients with oliguria and severe azotemia.[10]

CASE STUDY

3. What was the precipitating cause of the Henry's anemia?

OTHER DEFECTS AND DEFICIENCIES OF THE HMP SHUNT AND GSH METABOLISM

Erythrocytes synthesize about 50% of their total glutathione every 4 days. Congenital deficiencies of the enzymes needed for glutathione synthesis (glutathione synthetase, glutamylcysteine synthetase) have been reported to be associated with a decrease in GSH and a hereditary nonspherocytic hemolytic anemia. Hemolysis increases during administration of certain drugs. Deficiencies of glutathione

reductase, an enzyme that catalyzes the reduction of GSSG to GSH, although rare, have been reported.[34,35] Glutathione peroxidase catalyzes the detoxification of hydrogen peroxide by GSH. Deficiencies of this enzyme, although common, are not a cause of hemolysis. This might be explained by the fact that peroxide reduction by GSH occurs nonenzymatically at a significant rate.

PYRUVATE KINASE (PK) DEFICIENCY

Pyruvate kinase deficiency is the most common enzyme deficiency in the glycolytic pathway and the second most common erythrocyte enzyme deficiency. Although PK deficiency is less common than G6PD deficiency, it is the most common inherited enzyme defect associated with a *chronic* hemolytic anemia.[36] Many pyruvate kinase enzyme mutations account for the disorder's variability of clinical manifestations. The more severe types are noted in infancy, whereas the milder types may not be detected until adulthood. Inheritance is autosomal recessive. Clinically significant hemolytic anemias due to PK deficiency are associated with the homozygous state or double heterozygosity for two mutant enzymes. The variation in clinical phenotype associated with the genetic mutations appears to reflect not only the aberrant properties of the mutant protein but also interactions of the genotype with physiological and environmental factors including epigenetic modifications, ineffective erythropoiesis, splenic function, and coexisting polymorphisms of other enzymes.[37] Simple heterozygotes are usually asymptomatic. Acquired PK deficiency is seen in some leukemias and myelodysplastic disorders.

Pathophysiology

More than 220 different mutations in the PK gene, 178 of which result in PK deficiency (*PKLR* gene on chromosome 1q21) have been identified.[38] Most are missense mutations, but nonsense mutations, deletions, and insertions are also found. PK catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate, concurrent with the conversion of ADP to ATP (Figure 18-4 ■). PK deficiency compromises this energy-producing reaction, resulting in a decrease of ATP production. The cell's inability to maintain normal ATP levels results in alterations of the erythrocyte membrane: failure of the cation pumps causing potassium loss as well as sodium and calcium gain and dehydration (echinocytes). The echinocytes are sequestered in splenic cords and phagocytized by macrophages.

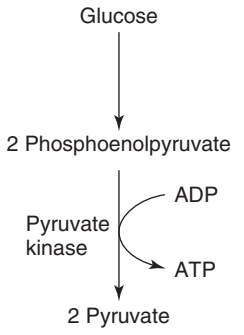
Clinical Findings

Clinical symptoms vary depending on the degree of anemia, which can vary from mild to severe. Individuals tolerate the anemia relatively well because of the increase in 2,3-BPG that accompanies this distal block in glycolysis. The two to three times normal increase in 2,3-BPG enhances the release of oxygen to the tissues. Jaundice can occur with intermittent hemoglobinuria. Gallstones are a common complication due to high levels of bilirubin excreted from the liver in the bile.

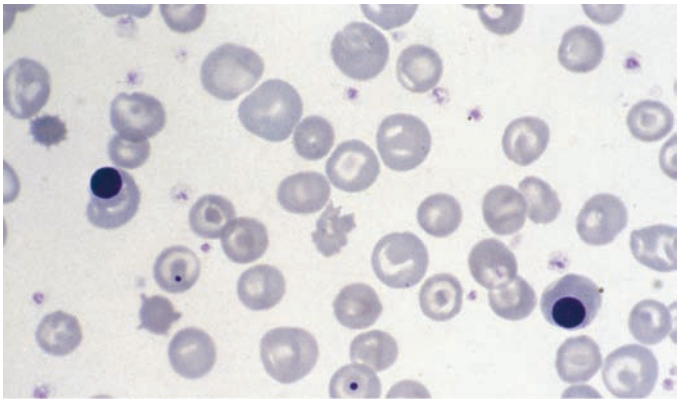
PK deficiency can be life threatening in neonates. When anemia is present at birth, PK deficiency should be considered and differentiated from other etiologies associated with anemia in newborns (ABO/Rh incompatibility, thalassemia, G6PD deficiency, and hereditary spherocytosis).[39] Severe PK deficiency can cause pronounced jaundice in neonates that may require exchange transfusions, and some cases have been associated with hydrops fetalis.[39,40] Splenectomy in older children can result in stabilization of the hemoglobin and decrease the need for transfusions. Patients with milder forms of PK deficiency are commonly diagnosed in early adulthood although they may have had neonatal jaundice.[21]

Laboratory Findings

Patients with PK deficiency have a normocytic, normochromic anemia with hemoglobin levels of 6–12 g/dL. Reticulocytosis ranges from 2–15% and increases after splenectomy, often >40%. The degree of reticulocytosis before splenectomy is not proportional to the degree of anemia as it is in most other hemolytic anemias because the spleen preferentially sequesters and removes the younger PK deficient erythrocytes. The blood smear exhibits irregularly contracted cells and occasional echinocytes before splenectomy; more are found following splenectomy (Figure 18-5 ■). In contrast to G6PD deficiency, Heinz bodies and spherocytes are not found in PK deficiency. Serum unconjugated indirect bilirubin and LD are increased, and haptoglobin is decreased or absent. Osmotic fragility is normal, but cells



■ **FIGURE 18-4** Glucose is metabolized to pyruvate in the glycolytic pathway. ATP is generated as phosphoenolpyruvate and is converted to pyruvate with a net gain of 2 ATP. Two molecules of pyruvate are formed from 1 glucose molecule. In PK deficiency, this reaction is slowed, resulting in deficient ATP production.



■ **FIGURE 18-5** Blood smear from a patient with PK deficiency. Note the echinocyte, acanthocyte, target cells, and irregularly contracted cells. Howell-Jolly bodies are also present (Wright-Giemsa stain, 1000× magnification).

demonstrate increased hemolysis when incubated at 37°C. Autohemolysis is increased at 48 hours and is not corrected with the addition of glucose but is corrected with the addition of ATP.

In performing enzyme tests for PK, the erythrocytes must be separated from leukocytes because leukocytes contain more PK than erythrocytes. Two genes located on chromosomes 15q22 and 1q21 encode for pyruvate kinase. The first locus produces PK active in muscle, leukocytes, platelets, and various other tissues. The second locus produces the PK active in erythrocytes. Thus, in erythrocyte PK deficiency (mutations of the second locus), only the erythrocytes are deficient; leukocytes are normal. The screening procedure is based on the disappearance of fluorescence as erythrocytes are incubated with phosphoenol-pyruvate (PEP), LD, ADP, and NADH.[41]

$$\text{PEP} + \text{ADP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP}$$

$$\underset{\text{Fluorescence}}{\text{Pyruvate} + \text{NADH} + \text{H}^+} \xrightarrow{\text{LD}} \underset{\text{No Fluorescence}}{\text{Lactate} + \text{NAD}^+}$$

Some mutant PK enzymes have normal activity at high substrate concentrations and abnormal activity at low substrate concentrations. A modification of this procedure has been developed to improve the interpretation of the endpoint.[42] In this modification, patient blood is frozen and thawed to ensure complete hemolysis of the specimen before testing.

Fluorescence in normal erythrocytes disappears in 30 minutes. In PK-deficient erythrocytes, fluorescence persists 45–60 minutes. The quantitative test for PK deficiency is performed in the same manner as the screening test except that the rate of disappearance of fluorescence is measured in a spectrophotometer at 340 nm. A rapid potentiometric method has also been developed to measure enzymatic activity by monitoring the change in pH in a reaction buffer during the conversion of the substrate to pyruvate. Because of the large number of mutations and the low incidence of PK deficiency, molecular methods were initially not widely used to detect the disease[43] but more recently have largely replaced enzymatic assays.[36]

Therapy

There is no specific therapy for PK deficiency. Transfusions help maintain the hemoglobin above 8–10 g/dL. Splenectomy can improve the hemoglobin level and decrease the need for transfusions in some affected individuals; however, hemolysis continues.

☑ CHECKPOINT 18-4

What are the differentiating characteristics of PK and G6PD deficiencies found on the peripheral blood smear?

OTHER ENZYME DEFICIENCIES IN THE GLYCOLYTIC PATHWAY

Other enzyme deficiencies in the glycolytic pathway, when associated with anemia, have clinical manifestations and laboratory findings that resemble those of PK deficiency.

- **Glucose phosphate isomerase deficiency (GPI)** This is the second most common disorder of the glycolytic pathway. Almost all GPI mutants are unstable, causing hemolytic anemia. Affected individuals show a partial response to splenectomy.
- **Hexokinase (HK) deficiency** As the first enzyme in the glycolytic pathway HK is responsible for priming the glycolytic pump. Of the two types of HK deficiency, one is associated with hemolytic anemia that responds to splenectomy. The other is associated not only with hemolytic anemia but also with an array of other abnormalities. Because the metabolic defect occurs before the generation of 2,3-BPG, production of 2,3-BPG is reduced and patients tolerate the anemia poorly.
- **Phosphoglycerokinase (PGK) deficiency** A sex-linked disorder, PGK deficiency causes hemolytic anemia and mental retardation in males, but females have a milder form of the disorder.
- **Phosphofructokinase (PFK) deficiency** This is indicated when subunits of the PFK enzyme are found in various tissues. Deficiency of this enzyme can appear as myopathy, hemolytic anemia, or both.[44]
- **Triosephosphateisomerase (TPI) deficiency** This causes severe abnormalities in erythrocytes resulting in severe hemolysis. Abnormalities are also noted in striated muscle and the central nervous system. Death in infancy is common.

ABNORMAL ERYTHROCYTE NUCLEOTIDE METABOLISM

Pyrimidine 5′-nucleotidase (P5N) contributes to the degradation of nucleic acids by cleaving pyrimidine nucleotides into smaller nucleosides that can diffuse out of the cell. The buildup of pyrimidine nucleotides decreases the adenine nucleotide pool needed for normal function. P5N deficiency is an autosomal recessive disorder that leads to a severe hemolytic anemia unresponsive to splenectomy. Partially degraded mRNA and rRNA accumulate within the cell and are visualized as basophilic stippling in stained smears. Lead inhibits this enzyme, which may explain the similar coarse basophilic stippling seen in lead poisoning (Chapter 12).

Summary

The erythrocyte life span can be significantly shortened if the erythrocyte has intrinsic defects such as deficient metabolic machinery. Maintaining a balance of intracellular constituents is compromised when enzymes responsible for various metabolic pathways are deficient or fail to function properly. These abnormalities are almost always inherited defects. Erythrocytes with intrinsic defects are susceptible to early destruction, and when this destruction exceeds the marrow capacity to replace cells, hemolytic anemia results. These hemolytic anemias are known as *chronic* or *hereditary nonspherocytic hemolytic anemias*.

Erythrocyte enzyme deficiencies can compromise the integrity of the cell membrane or hemoglobin and lead to hemolysis. The HMP shunt provides the cell-reducing power, protecting it from oxidant damage. Defects in this shunt allow hemoglobin to be oxidized and denatured to Heinz bodies. The Heinz bodies damage the cell membrane. The finding of bite cells (degmacytes) on the blood smear is evidence that Heinz bodies have been pitted from the cells. The most common deficiency in this pathway is G6PD deficiency, a sex-linked disorder. This enzyme has many different variants, some of which cause severe hemolysis and others mild hemolysis. In most cases, hemolysis is sporadic, occurring during infections or with the administration of certain drugs, and is self-limited. In these variants, the younger cells have adequate enzyme activity, but the older cells are severely deficient and selectively hemolyzed. Testing for the enzyme should be delayed until 2 months after the hemolytic episode when reticulocytes are at a steady state (normal) and the erythrocytes produced after the hemolytic episode have aged. Screening tests for the enzyme include the fluorescent dye test, cytochemical staining, dye reduction test, and ascorbate cyanide test. Definitive testing requires quantitation of the enzyme or genetic testing for mutations.

Deficiencies of enzymes in the glycolytic pathway decrease ATP production and lead to hemolysis. Cation pump activity can be impaired and osmotic fragility can be increased. Pyruvate kinase (PK) is the most common enzyme abnormality in this pathway. Many PK enzyme mutants exist, resulting in a diverse array of clinical and laboratory findings. Reticulocytosis can be significant. The blood film is remarkable for the presence of irregularly contracted cells and echinocytes. Heinz bodies and bite cells are not found. Screening and definitive tests for the enzyme are based on fluorescence. Several other rare enzyme defects that also lead to hemolysis have been identified.

Review Questions

Level I

1. What are the two main metabolic pathways that erythrocytes use for glucose metabolism? (Objective 1)
   A. Krebs cycle and glycolytic pathway
   B. hexokinase and Krebs cycle
   C. oxidative phosphorylation and glycolytic pathway
   D. hexosemonophosphate shunt and glycolytic pathway
2. The main protective functions of erythrocyte enzymes result from which of the following? (Objective 2)
   A. electron transport and cation pumping using ATP
   B. cation pumping using ATP and protection of hemoglobin by reduced glutathione
   C. protection of hemoglobin by reduced glutathione and electron transport
   D. cation pumping and bilirubin production
3. In G6PD deficiency, anemia ultimately results from: (Objective 2)
   A. buildup of 2,3-BPG and poor iron binding
   B. inability to maintain enough ATP to pump cations
   C. oxidative damage to hemoglobin and splenic removal of erythrocytes
   D. membrane protein defects and loss of erythrocyte flexibility
4. Which is the most common erythrocyte enzyme deficiency? (Objective 3)
   A. pyruvate kinase
   B. hexokinase
   C. glucose phosphate isomerase
   D. glucose-6-phosphate dehydrogenase
5. Which is true for the inheritance pattern for G6PD? (Objective 4)
   A. It is X-linked and only found in males.
   B. It is autosomal dominant and affects all offspring.
   C. It is X-linked; however, females can be affected.
   D. It is autosomal, and males are affected; females are carriers.
6. Which of the following is a quantitative test for G6PD? (Objective 6)
   A. rate reduction test
   B. fluorescent spot test
   C. dye reduction test
   D. ascorbate cyanide test

7. Following a hemolytic episode, which of the following is a common finding in G6PD deficiency? (Objective 5)
   A. reticulocytosis
   B. appearance of burr cells on the blood smear
   C. increased haptoglobin
   D. decreased unconjugated bilirubin
8. An abnormal erythrocyte resulting from splenic removal of Heinz bodies in erythrocytes is called: (Objective 7)
   A. macrocyte
   B. target cell
   C. bite cell
   D. dacryocyte
9. Following a hemolytic episode in a G6PD-deficient individual, a characteristic finding on a blood smear is: (Objective 7)
   A. increased polychromasia
   B. echinocytes
   C. Howell-jolly bodies
   D. Pappenheimer bodies
10. What compound can induce anemia in G6PD deficiency? (Objective 8)
    A. aspirin
    B. vitamin C
    C. iron
    D. primaquine

Level II

1. Why should G6PD testing be delayed for an individual following a hemolytic episode? (Objective 1)
   A. The level of glucose must have time to replenish.
   B. Heinz bodies can interfere with the test method.
   C. Deficient cells have been selectively destroyed.
   D. The patient needs time to build up iron stores.
2. An 18-year-old black male was suspected of having G6PD deficiency when he experienced hemolytic anemia after administration of primaquine. An erythrocyte G6PD analysis performed on blood taken 2 days after symptoms appeared was normal. A reticulocyte count revealed 12% reticulocytes at this time. These results suggest that: (Objective 7)
   A. the patient definitely does not have G6PD deficiency but could have pyruvate kinase deficiency
   B. another G6PD test should be done in several months when the reticulocyte count returns to normal
   C. leukocytes could be contaminating the sample, giving a false result
   D. the patient probably has the G6PD-Mediterranean variant
3. The main consequence of enzyme defects in the glycolytic pathway is: (Objective 3)
   A. decreased ATP production
   B. Heinz body formation
   C. decreased formation of reduced glutathione
   D. decreased formation of 2,3-BPG
4. Which of the following G6PD isoenzyme variants will *not* result in hemolysis? (Objective 4)
   A. G6PD-Mediterranean
   B. G6PD-B
   C. G6PD-Canton
   D. G6PD-A^{-}
5. The blood smear of a patient with chronic nonspherocytic hemolytic anemia reveals echinocytes, acanthocytes, target cells, and irregularly contracted cells. Which follow-up test could help define the cause of this anemia? (Objective 6)
   A. PEP fluorescent test
   B. ascorbate cyanide test
   C. fluorescent spot test
   D. quantitation of G6PD
6. Explain why bite cells are *not* characteristic of pyruvate kinase deficiency. (Objectives 3, 5, 6)
   A. The spleen removes them as they are formed.
   B. The erythrocyte forms a spherocyte as inclusions are removed.
   C. Heinz bodies are not formed in pyruvate kinase deficiency.
   D. Pyruvate kinase deficiency does not cause abnormal erythrocyte morphology.
7. Hereditary nonspherocytic hemolytic anemia syndrome is associated with which G6PD variants? (Objective 4)
   A. class I
   B. class II
   C. class III
   D. class IV
8. Harmful peroxides in the erythrocyte are neutralized by which of the following? (Objective 2)
   A. ATP production
   B. Heinz bodies
   C. bilirubin
   D. glutathione

9. A patient is suspected of having a form of G6PD deficiency but is *not* affected by ingestion of fava beans. How can this be explained? (Objective 4)
   A. The patient has the G6PD-Mediterranean type.
   B. The G6PD-B type of G6PD is present.
   C. The patient does not have G6PD deficiency.
   D. A mild form of G6PD is present.

10. Which lab test is most useful in diagnosing G6PD deficiency? (Objective 1)
   A. fluorescent spot test
   B. CBC
   C. reticulocyte count
   D. PEP fluorescent test

Companion Resources

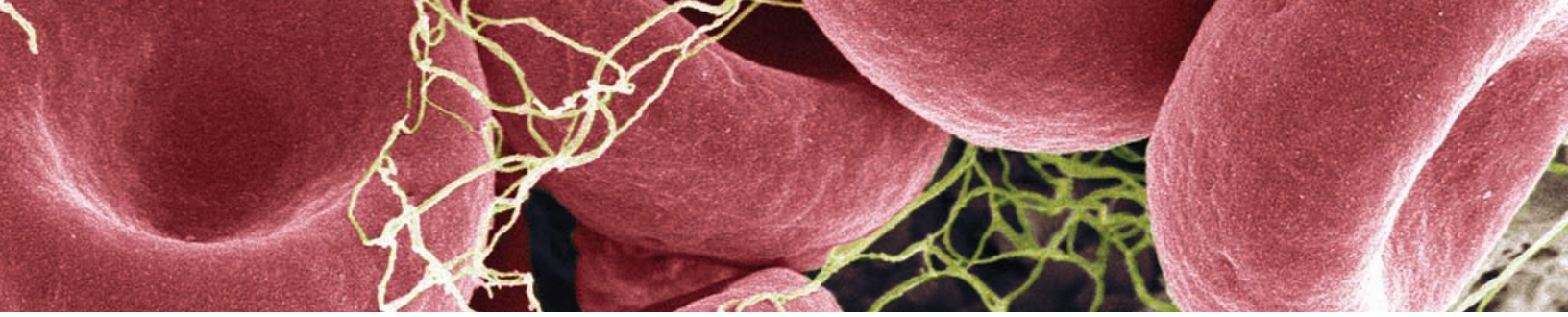
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. van Solinge WW, van Wijk R. Disorders of red cells resulting from enzyme abnormalities. In: Lichman MA, Kipps TJ, Seligsohn U et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill Medical; 2010:647–74 (available at www.accessmedicine.com).
2. Herz F, Kaplan E, Scheye ES. Diagnosis of erythrocyte glucose-6-phosphate dehydrogenase deficiency in the Negro male despite hemolytic crisis. *Blood*. 1970;35:90.
3. Ringelhahn B. A simple laboratory procedure for the recognition of A- (African type) G6PD deficiency in acute haemolytic crisis. *Clin Chim Acta*. 1972;36:772.
4. Department of the Army, Memorandum for Commanders, MEDCOM major subordinate commands, Army glucose 6-phosphate dehydrogenase (G6-PD) deficiency screening program, February 18, 2004.
5. Price EA, Otis S, Schrier SL. Red blood cell enzymopathies. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier; 2013:581–91.
6. Luzzatto L, Usanga EA, Reddy S. Glucose-6-phosphate dehydrogenase deficient red cells: resistance to infection by malarial parasites. *Science*. 1969;164:839–42.
7. Cappadoro M, Giribaldi G, O'Brien E et al. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by plasmodium falciparum can explain malaria protection in G6PD deficiency. *Blood*. 1998;92:2527–34.
8. Carter N, Pamba A, Duparc S et al. Frequency of glucose-6-phosphate dehydrogenase deficiency in malaria patients from six African countries enrolled in two randomized anti-malarial clinical trials. *Malar J*. 2011;10:241.
9. Guindo A, Fairhurst RM, Doumbo OK et al. X-Linked G6PD deficiency protects hemizygous males but not heterozygous females against severe malaria. *PLoS Med*. 2007;4(3):e66.
10. Bienzle U, Lucas A, Ayeni O et al. Glucose-6-phosphate dehydrogenase and malaria: greater resistance of females heterozygous for enzyme deficiency and of males with non-deficient variant. *Lancet*. 1972;299(7742):107–10.
11. Arese P, DeFlora A. Pathophysiology of hemolysis in glucose-6-phosphate dehydrogenase deficiency. *Semin Hematol*. 1990;27:1–40.
12. WHO Working Group. Glucose-6-phosphate dehydrogenase deficiency. *Bull World Health Organ*. 1989;67(6):601–11.
13. Costa E, Cabeda JM, Vieira E et al. Glucose-6-phosphate dehydrogenase averia: a de novo mutation associated with chronic nonspherocytic hemolytic anemia. *Blood*. 2000;95:1499–501.
14. Darr S, Vulliamy TJ, Kaeda J et al. Molecular characterization of G6PD deficiency in Oman. *Hum Hered*. 1996;46:172–76.
15. Sarkar S, Prakash D, Marwaha RK et al. Acute intravascular haemolysis in glucose-6-phosphate dehydrogenase deficiency. *Ann Trop Paediatr*. 1993;13:391–94.
16. Arese P, Turrini F, Schwarzer E. Band3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cell Physiol Biochem*. 2005;16:133–46.
17. Nafa L, Reghis A, Osmani N et al. G6PD Aures: a new mutation (48 Ile → Thr) causing mild G6PD deficiency is associated with favism. *Hum Mol Genet*. 1993;2:81–82.
18. Vives Corrons JS, Feliu E, Pujades MA et al. Severe glucose-6-phosphate dehydrogenase (G6PD) deficiency associated with chronic hemolytic anemia, granulocyte dysfunction, and increased susceptibility to infections: description of a new molecular variant (G6PD Barcelona). *Blood*. 1982;59:428–34.
19. Roos D, van Zwieten R, Wijnen JT et al. Molecular basis and enzymatic properties of glucose-6-phosphate dehydrogenase volendam, leading to chronic nonsperocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. *Blood*. 1999;94:2955–62.
20. Gray GR, Klebanoff SJ, Stamatoyannopoulus G et al. Neutrophil dysfunction, chronic granulomatous disease and non-sperocytichaemolytic anemia caused by complete deficiency of glucose-6-phosphate dehydrogenase. *Lancet*. 1973;2:530–34.
21. Wolach B, Ashkenazi M, Grossmann R et al. Diurnal fluctuation of leukocyte G6PD activity: a possible explanation for the normal neutrophil bactericidal activity and the low incidence of pyogenic infections in patients with severe G6PD deficiency in Israel. *Pediatr Res*. 2004;55:807–13.
22. Theodorsson E, Birgens H, Hagve TA. Haemoglobinopathies and glucose-6-phosphate dehydrogenase deficiency in a Scandinavian perspective. *Scand J Clin Lab Invest*. 2007;67(1):3–10.
23. Johnson LH, Bhutani VK, Brown AK. System-based approach to management of neonatal jaundice and prevention of kernicterus. *J Pediatr*. 2002;140:396–403.
24. Mesner O, Hammerman C, Goldschmidt D et al. Glucose-6-phosphate dehydrogenase activity in male premature and term neonates. *Arch Dis Child Fetal Neonatal Ed*. 2004;89:F555–57.
25. Ward PCJ, Schwartz BS, White JG. Heinz-body anemia: "bite cell" variant—a light and electron microscopic study. *Am J Hematol*. 1983;15:135–46.
26. Simmons SJ, McCann S, Glassman AB. Intracorpuscular defects II: hereditary enzyme deficiencies. In: Harmening D., ed. *Clinical Hematology and Fundamentals of Hemostasis*, 3rd ed. Philadelphia: F.A. Davis; 1997:164–72.
27. Shannon K, Buchanan GR. Severe hemolytic anemia in black children with glucose-6-phosphate dehydrogenase deficiency. *Pediatrics*. 1982;70(3):364–69.
28. Nadarajan V, Shanmugam H, Sthaneshwar P et al. Modification to reporting of qualitative fluorescent spot test results improves detection of glucose-6-phosphate dehydrogenase (G6PD)-deficient heterozygote female newborns. *Int J Lab Hematol*. 2011;33:463–70.
29. Wong FL, Boo NY, Ainoon O et al. Comparison of detection of glucose-6-phosphate dehydrogenase deficiency using fluorescent spot test, enzyme assay and molecular method for prediction of severe neonatal hyperbilirubinaemia. *Singapore Med J*. 2009:5062–67.
30. Kim S, Nguon C, Guillard B et al. Performance of the CareStart™ G6PD deficiency screening test, a point-of-care diagnostic for primaquine therapy screening. *PLoS One*. 2011;6(12):e28357.

31. Tinley KE, Loughlin AM, Jepson A et al. Evaluation of a rapid qualitative enzyme chromatographic test for glucose-6-phosphate dehydrogenase deficiency. *Am J Trop Med Hyg*. 2010;82:210–14.
32. Van Noorden CJ, Dolbeare F, Aten J. Flow cytofluorometric analysis of enzyme reactions based on quenching of fluorescence by the final reaction product: detection of glucose-6-phosphate dehydrogenase deficiency in human erythrocytes. *J Histochemy Cytochem*. 1989;37(9):1313–18.
33. Miao J-K, Chen Q-X, Bao L-M et al. Determination of optimal cutoff value to accurately identify glucose-6-phosphate dehydrogenase-deficient heterozygous female neonates. *Clin Chim Acta*. 2013;424:131–35.
34. Loos H, Roos D, Weening R et al. Familial deficiency of glutathione reductase in human blood cells. *Blood*. 1976;48:53–72.
35. Kamerbeek NM, van Zwieten R, de Boer M et al. Molecular basis of glutathione reductase efficiency in human blood cells. *Blood*. 2007;109:3560–66.
36. Gregg XT, Prchal JT. Red blood cell enzymopathies. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Procedures*, 4th ed. New York: Churchill Livingstone; 2005:611–22.
37. Zanella A, Fermo E, Bianchi P et al. Pyruvate kinase deficiency: the genotype-phenotype association. *Blood Rev*. 2007;21(4):217–31.
38. The Human Gene Mutation Database, at the Institute of Medical Genetics, Cardiff (available at www.hgmd.cf.ac.uk [input gene, *PKLR*]; accessed September 12, 2013).
39. Pissard S, de Montalembert M, Bachir D et al. Pyruvate kinase (PK) deficiency in newborns: the pitfalls of diagnosis. *J Pediatr*. 2007;150(4):443–45.
40. Hennekam L, Beemer FA, Cats BP et al. Hydrops fetalis associated with red cell pyruvate kinase deficiency. *Genet Couns*. 1990;1:75–79.
41. Beulter E. A series of new screening procedures for pyruvate kinase, glucose-6-dehydrogenase deficiency and glutathione reductase deficiency. *Blood*. 1996;28:553.
42. Tsang SS, Feng CS. A modified screening procedure to detect pyruvate kinase deficiency. *Am J Clin Pathol*. 1993;99:128–31.
43. Whitney KM, Lothrop CD Jr. Genetic test for pyruvate kinase deficiency of Basenjis. *J Am Vet Med Assoc*. 1995;207:918–21.
44. Vora S, Corash L, Engel WK et al. The molecular mechanism of the inherited phosphofructokinase deficiency associated with hemolysis and myopathy. *Blood*. 1980;55:629–35.



19 Hemolytic Anemia: Immune Anemias

LINDA A. SMITH, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. List the antibody systems or specificities usually involved in:
   a. cold agglutinin syndrome
   b. paroxysmal cold hemoglobinuria (PCH)
   c. warm autoimmune hemolytic anemias (WAIHA)
2. Describe the purpose and general procedure for the direct antiglobulin test (DAT) and identify the typical DAT profile (polyspecific AHG, anti-IgG, anti-C3) found in patients with cold agglutinin syndrome and warm autoimmune hemolytic anemia.
3. Describe and recognize the characteristic hematologic findings associated with the following conditions:
   a. cold agglutinin syndrome
   b. warm autoimmune hemolytic anemia (WAIHA)
4. Describe and recognize the typical hematologic laboratory findings in ABO and Rh hemolytic disease of the fetus and newborn (HDFN).
5. Contrast alloimmune and autoimmune hemolytic anemias including stimulus for antibody production, site of hemolysis, type of antibody involved, and direct antiglobulin test (DAT) and indirect antiglobulin test (IAT) reactions.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Compare the pathophysiology of extravascular and intravascular hemolysis in immune hemolytic anemia (IHA) including site of destruction, immunoglobulin class of antibody, and underlying mechanism.
2. Given a set of laboratory data, determine the underlying mechanism of hemolysis, and suggest confirmatory tests.
3. Contrast the different mechanisms of drug-induced immune hemolytic anemia and tests used for confirmation, and identify the drugs commonly involved.
4. Describe the mechanism of hemolysis, select the confirmatory tests for paroxysmal cold hemoglobinuria (PCH), and evaluate the results.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

5. Compare the prenatal and postnatal pathophysiology of hemolytic disease of the fetus and newborn (HDFN).
6. Interpret the results of laboratory tests for HDFN, and determine whether evidence of hemolysis is present.
7. Compare and contrast the pathophysiology and clinical findings of an immediate transfusion reaction with those of a delayed reaction.
8. List the causes of the secondary types of cold agglutinin syndrome and warm autoimmune hemolytic anemia and identify key laboratory results linking the cause and the autoimmune condition.

Key Terms

Alloimmune hemolytic anemia
Antihuman globulin (AHG)
Autoimmune hemolytic anemia (AIHA)
Cold agglutinin syndrome (CAS)
Direct antiglobulin test (DAT)
Donath-Landsteiner (D-L) antibody
Drug-induced hemolysis
Erythroblastosis fetalis
Hemolytic disease of the fetus and newborn (HDFN)
Hemolytic transfusion reaction
Immune hemolytic anemia (IHA)
Immune tolerance
Indirect antiglobulin test (IAT)
Kernicterus
Paroxysmal cold hemoglobinuria (PCH)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. Additionally, a basic understanding of immunology principles and an introduction to immunohematology will be helpful. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Summarize the normal production, life span, and destruction of erythrocytes. (Chapter 5)
- List the reference interval for the hematology parameters: hemoglobin, hematocrit, erythrocyte count, and reticulocytes. (Chapters 10,11)
- List the different intrinsic and extrinsic factors that can result in hemolytic anemia. (Chapter 11)
- Describe the basic structure of immunoglobulin and its normal physiologic role. (Chapter 8)

Level II

- Describe the role of erythropoietin and regulation of its production. (Chapter 5)
- Differentiate intravascular and extravascular destruction of erythrocytes and relate these types of destruction to laboratory parameters in the diagnosis of abnormal hemolysis. (Chapters 5, 11)
- Choose laboratory tests to assist in diagnosing anemia in a cost efficient and effective manner. (Chapter 11)
- Identify clinical signs of hemolytic anemia and changes that occur in laboratory tests that signal possible hemolytic anemia. (Chapter 11)

CASE STUDY

We will refer to this case study throughout the chapter.

Nancy, a 28-year-old female, makes an appointment with her physician because she feels tired all the time and is short of breath with minor exertion. She indicates that the symptoms have been ongoing for about 3 weeks. She has no known history of chronic diseases.

Consider the initial laboratory tests that should be performed to evaluate this patient's condition based on clinical history and symptoms.

OVERVIEW

This chapter compares the different types of immune-mediated hemolytic anemias: autoimmune, alloimmune, and drug induced. The underlying mechanism for each of these anemias involves the reaction of an antibody with erythrocyte antigens and subsequent cell destruction by either intravascular or extravascular processes. The chapter also compares mechanisms of intravascular and extravascular hemolysis and describes the tests necessary to detect erythrocyte sensitization and identify the causative antibody.

The chapter discusses pathophysiology, clinical and laboratory findings, differential diagnosis, and therapy for each of the major types of autoimmune hemolytic anemia. Hemolytic transfusion reactions and hemolytic disease of the fetus and newborn (HDFN) are included as examples of alloimmune hemolytic anemia. The causative antibody and clinical presentation for acute and delayed transfusion reactions are compared and the laboratory tests required to confirm hemolytic transfusion reactions are described. The section on HDFN compares ABO-HDFN and Rh-HDFN. The treatment of a fetus and a newborn with this condition as well as preventative measures are included.

INTRODUCTION

When an immune-mediated process (antibody and/or complement) destroys erythrocytes prematurely, the disorder is referred to as an **immune hemolytic anemia (IHA)**. The individual, however, might or might not become anemic, depending on the severity of hemolysis and the ability of the bone marrow to compensate for erythrocyte loss. Diagnosis of anemia (and underlying hemolysis) is determined by laboratory findings such as a decrease in hemoglobin and hematocrit, an increase in reticulocytes and/or unconjugated bilirubin, and a decrease in serum haptoglobin. Initial confirmation of the underlying immune mechanism is accomplished by demonstrating attachment of antibody or complement to the patient's erythrocytes. Table 11-14 (Chapter 11) summarizes some common laboratory values characteristically seen in hemolytic anemia.

CLASSIFICATION OF IMMUNE HEMOLYTIC ANEMIAS

Determining the underlying process of immune hemolysis is important because each type requires a specific treatment regimen. Initially, IHA can be classified into three broad categories based on the stimulus for antibody production[1,2] (Table 19-1 ★). These are:

- Autoimmune hemolytic anemia
- Drug-induced hemolytic anemia
- Alloimmune hemolytic anemia

Autoimmune hemolytic anemia (AIHA) is a complex and incompletely understood process characterized by an immune reaction against self-antigens and shortened erythrocyte survival.[1,2] Individuals produce antibodies against their own erythrocyte antigens (autoantibodies), which are usually directed against high-incidence antigens (antigens present on the erythrocytes of most people). The autoantibodies characteristically react not only with the individual's own erythrocytes but also with the erythrocytes of other individuals carrying that antigen. The reactions that occur with autoantibodies include sensitization (attachment of antibody or complement to the erythrocytes), agglutination of the erythrocytes, or erythrocyte lysis.

Autoimmune hemolytic anemias are further classified as warm or cold hemolytic anemia based on clinical symptoms and on the optimal temperature at which the antibody reacts in vivo and in vitro[1–3] (Table 19-2 ★). Some antibodies react best at body temperature (37°C); the anemia they produce is termed *warm autoimmune hemolytic anemia* (*WAIHA*). About 70% of the AIHAs are of the warm type. The majority of warm autoantibodies are of the IgG class (most frequently IgG1) and cause extravascular hemolysis of the erythrocyte. A few warm-reacting autoantibodies of either the IgM or IgA class have been identified.[4–6] Cold hemolytic anemias, on the other hand, are usually due to the presence of an IgM antibody with an optimal thermal reactivity below 30°C. Hemolysis with cold-reacting antibodies results from IgM binding to and activating complement. The IgM antibody attaches to erythrocytes in the cold and fixes complement. After warming, the antibody dissociates from the cell, but the complement remains, either causing direct cell lysis or initiating extravascular destruction. Included in the cold hemolytic anemia classification is a special condition, *paroxysmal cold hemoglobinuria (PCH)*, which is characterized by a cold-reacting IgG antibody capable of fixing complement.

A third category, *mixed-type autoimmune hemolytic anemia*, demonstrates both warm-reacting (IgG) autoantibodies and cold-reacting (IgM) autoantibodies.

★ **TABLE 19-1** Classification of Immune Hemolytic Anemias

Classification	Causes
Autoimmune	• Warm-reactive antibodies (37°C)
	Primary or idiopathic
	Secondary
	• Autoimmune disorders (systemic lupus erythematosus, rheumatoid arthritis, and others)
	• Chronic lymphocytic leukemia and other immunoproliferative diseases
	• Viral infections
	• Neoplastic disorders
	• Chronic inflammatory diseases
	• Cold-reactive antibodies (<30°C)
	Primary or idiopathic (cold hemagglutinin disease)
	Secondary
	• Infectious diseases (*Mycoplasma pneumoniae*, Epstein-Barr virus, other organisms)
	• Lymphoproliferative disorders
	Paroxysmal cold hemoglobinuria
	• Idiopathic
	• Secondary
	Viral syndromes
	Syphilis (tertiary)
	• Mixed type
	• DAT negative
Drug induced	• Drug dependent
	• Drug independent
	• Nonimmunologic protein adsorption (NIPA)
Alloimmune	• Hemolytic transfusion reaction
	• Hemolytic disease of the fetus and newborn

★ **TABLE 19-2** Characteristics of Agglutinins in Hemolytic Anemia

	Warm-Reacting Antibodies	Cold-Reacting Antibodies
Immunoglobulin (Ig) class	IgG	IgM
	IgM (rare)	IgG (PCH only)
	IgA (usually with IgG)	
Optimal reactivity	37°C	<30°C, usually <10°C
Mechanism of hemolysis	Extravascular	Intravascular: complement-mediated lysis
	Attachment of membrane-bound IgG or C3b to macrophage receptors	Extravascular: attachment of membrane-bound C3b to macrophage receptors
Specificity	Usually broad specificity anti-Rh	Usually autoanti-I
		Occasionally autoanti-i
		PCH: autoanti-P

PCH = paroxysmal cold hemoglobinuria

Drugs that attach to the erythrocyte membrane or alter it in some way can cause **drug-induced hemolysis**. Historically, several different mechanisms of hemolysis have been hypothesized based on whether the drug binds directly to the cell, reacts with an antibody in the circulation to form an immune complex that binds to the cell, or alters the erythrocyte antigens to stimulate formation of autoantibodies. Now however these antibodies are broadly classified as either drug dependent or drug independent based on reactions of patient's erythrocytes and the drug in in vitro test systems.[2,7] These mechanisms are discussed in detail later in this chapter.

AIHA occurs as a result of antibody development to an erythrocyte antigen that the individual lacks. When an individual is exposed to erythrocytes from another person, there could be antigens on the transfused cells that are not present on the recipient's erythrocytes. Therefore, the recipient's lymphocytes recognize antigens on the transfused cells as foreign and stimulate the production of antibodies (alloantibodies). In contrast to autoantibodies, these alloantibodies react only with the antigens on the transfused cells or cells from individuals who possess the antigen. The alloantibodies do not react with the individual's own erythrocytes. Examples of alloimmune hemolytic anemia are:

- HDFN in which the mother makes antibodies against antigens on the fetal erythrocytes
- Transfusion reactions in which the recipient makes antibodies to antigens on the transfused (donor) cells

The presence of alloantibodies can be detected in vitro by performing an antibody screen in which the patient's serum reacts with commercial erythrocytes containing most of the clinically significant antigens. An autocontrol consisting of the patient's serum and erythrocytes can also be set up. When only alloantibodies are present, the autocontrol shows no hemolysis or agglutination, whereas the mixture of patient's serum and the commercial cells produce agglutination and, in rare cases, hemolysis.

☑ CHECKPOINT 19-1

What are the three major categories of immune hemolytic anemia, and how is antibody production stimulated in each type?

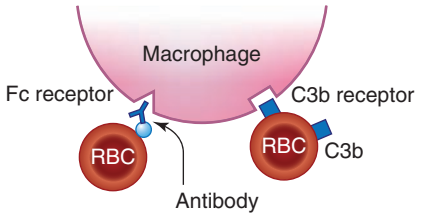
CASE STUDY (continued from page 349)

Nancy's initial complete blood count (CBC) shows a hemoglobin value of 7.0 g/dL and a hematocrit of 21%. Her white blood cell (WBC) count and platelet count are within the reference interval.

1. What are some reasons for her to have a low hemoglobin value?

SITES AND FACTORS THAT AFFECT HEMOLYSIS

Regardless of whether it is caused by alloantibodies or autoantibodies, hemolysis can be intravascular or extravascular, depending on the class of antibody involved and whether the complement cascade has been completely activated. Most immune-mediated hemolysis is extravascular. Erythrocytes sensitized (coated) with antibody (IgG) or complement components (e.g., C3b) attach to macrophages in the spleen or liver via macrophage receptors for the Fc portion of IgG or the C3b component of complement. These cells are then phagocytized (Figure 19-1 ■). Intravascular hemolysis occurs if the complement cascade is activated through C9 (the membrane attack complex), resulting in lysis of the cell. The rate at which hemolysis occurs in hemolytic anemia is related to several factors.[8–11] Table 19-3 ★ summarizes the major factors and their effects. A full description of the mechanisms is found on the Web (Additional Materials: Factors Affecting Hemolysis).



■ **FIGURE 19-1** Immune-mediated extravascular hemolysis. Erythrocytes sensitized with antibody or complement (C3b) attach to macrophages via specific cell receptors for these immune proteins.

★ **TABLE 19-3** Factors Affecting the Rate of Hemolysis in Immune Hemolytic Anemias

Factor	Effect
Class of immunoglobulin coating the erythrocytes	For IgG subclasses, the affinity of macrophage receptors and rate of hemolysis is greatest for IgG3 and IgG1
Quantity of Ig molecules per erythrocyte	Immunoglobulins coat a cell with a high density (large number) of corresponding antigens more heavily than a cell with low density (fewer) antigens
Ability of the Ig to activate complement	IgM and IgG can activate complement; for IgG, the ability to activate complement is IgG3 > IgG1 > IgG2
Thermal amplitude of the antibody	Warm-reacting (37°C) antibodies can cause hemolysis but cold-reacting (0–4°C) antibodies usually do not
The activity level of macrophages	Suppression of Fc receptors decreases rate of hemolysis
Complement components on the membrane	Macrophages have receptors for C3b but no effective receptor for C3d

☑ CHECKPOINT 19-2

Explain how the class of immunoglobulin, amount of antibody bound, and thermal reactivity of the antibody affect hemolysis.

MECHANISMS OF HEMOLYSIS

The mechanism for hemolysis is based on whether IgM, IgG, and/or complement are/is present on the erythrocyte. Specific phagocytic cells in the spleen or liver initiate extravascular hemolysis of cells coated with IgG or complement. Complete activation (through C9) of the complement cascade results in intravascular hemolysis.

IgG-Mediated Hemolysis

IgG mediates erythrocyte destruction by first attaching to the erythrocyte membrane antigens through the Fab portion of the Ig molecule. The Fc portion of the bound IgG is exposed and binds to Fc receptors (FcγR-I, -II, -III) on macrophages in the red pulp of the spleen. After binding, the macrophage pits the antigen–antibody (Ag/Ab) complex, fragmenting the cell membrane, which then reseals itself. With repeated splenic passage, the erythrocyte continues to lose membrane and gradually assumes a spherocytic shape. As the cell becomes more spherocytic, it becomes rigid and less deformable and is eventually phagocytized by splenic macrophages. Alternatively, the antibody-sensitized cell can be entirely engulfed by the macrophages (phagocytosis). Natural killer (NK) cells and neutrophils also have FcγR. Neutrophils are capable of phagocytosis (FcγR-I,-III). Interaction of antibody-coated cells with NK cells usually results in the recognition and lysis of cells by NK cells, which is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). This occurs through binding of IgG by the FcγRIII (CD16) on NK cells.

As the spleen becomes saturated with sensitized red blood cells (RBCs) (RBCs coated with antibody and/or complement), the liver assists in removing the cells. Lightly coated cells are more efficiently removed in the spleen due to the sluggish splenic blood flow. The liver can be of some importance in removing heavily sensitized cells. The splenic tissue proliferates in response to an increase in erythrocyte sequestration and can be responsible for splenic enlargement (splenomegaly) in chronic warm-immune hemolytic anemias (Chapter 3). In immune hemolytic anemias in which complement is activated, it as well as IgG is present on the erythrocyte membrane, which enhances phagocytosis by increasing the likelihood of the cell binding to either the Fc and/or C3b receptors of macrophages.

Complement-Mediated Hemolysis

The complement system consists of >20 serum proteins responsible for a number of diverse biological activities including the mediation of acute inflammatory responses and destruction of cells and microorganisms. The major roles of complement in immune hemolytic anemias are sensitization and lysis of erythrocytes.[11,12] Sensitization occurs when only a portion of the cascade is activated and deposited on the erythrocyte membrane; lysis results when the entire system is activated.

The complement proteins are designated numerically (e.g., C1, C2, C3) or by letters or historical names. Complement proteins normally circulate in an inactive state but under certain circumstances become activated in a cascadelike fashion. An activated component is identified by placing a bar over the component ($\overline{C5}$).

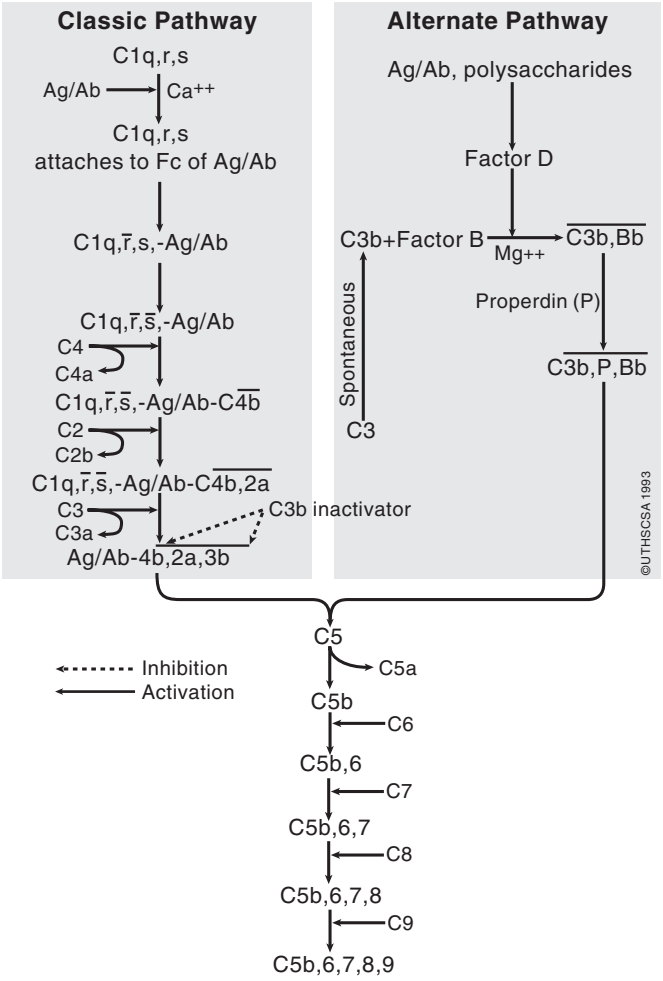
The complement cascade can be initiated by at least three separate mechanisms:

- Classic
- Alternate
- Lectin

The usual and most important activation pathway in IHA is the classic, although on some occasions the alternate pathway can be activated (Figure 19–2 ■). The classic complement pathway is initiated by an antigen–antibody reaction involving IgM as well as IgG1 and IgG3 antibodies (IgG2 antibodies activate but less efficiently). The first complement component (C1q) must attach to two antibody Fc regions, requiring that two antibody-binding sites be in close proximity. Thus, the attachment of complement depends on the density or concentration of antibody molecules and their spatial arrangement when attached to antigens on the cell surface. Only one IgM molecule (a pentameric structure) is required to activate the complement pathway, whereas two IgG molecules (monomeric) are required; as a result, IgM is much more likely to activate complement than IgG. Therefore, the nature of the antibody involved is an important determinant of the extent of erythrocyte destruction by complement.

Activation of the complement cascade is initiated when the first complement component, C1, binds to two Fc regions of IgG or IgM antibody molecules. This attachment initiates activation of the other complement components (C4, C2, C3). The complex containing C3b activates the terminal components, C5 to C9. Activation of these terminal complement components, known as the *membrane attack complex (MAC)*, is responsible for the lytic attack on the erythrocyte membrane. Membrane leakage begins at the C8-activation stage when a transmembrane pore is formed. C9 prevents the pore from resealing, resulting in osmotic lysis. The alternate pathway of complement activation, which bypasses C1, C2, and C4 and activates C3 directly, can be initiated by aggregated IgG, IgA, and IgE as well as by a number of polysaccharides and liposaccharides.

If complement activation on the erythrocyte membrane is complete (C1 → C9), intravascular hemolysis occurs. However, activation of complement does not always go through C9 and thus does not always lead to direct cell lysis. More commonly, activation proceeds only through C3 on the erythrocyte membrane (in which case the cell is said to be *sensitized*). As C3 is activated, it is broken down into two fragments: C3a, which is released into the plasma, and C3b, which remains attached to the cell membrane. The sensitized cell with attached C3b is totally or partially engulfed by binding to the C3b receptors (CR1, CR3) of macrophages in the liver (most complement-coated cells are removed in this organ). Because of the enzymatic action of the protein C3b inactivator in plasma, C3b on erythrocytes can be further cleaved to form C3c and C3d before the cell encounters



■ **FIGURE 19-2** The complement cascade. The central event in complement activation is the activation of C3 by C3 convertases. This can occur by two separate but interrelated mechanisms, the classic and alternate pathways. The classic complement pathway is initiated by an antigen–antibody reaction. The antigen–antibody complex activates the C1q, r, s complex, which in turn activates C4 by proteolytic cleavage to C4a and C4b. C2 binds to C4b and is proteolytically cleaved by C1s to form C2a and C2b. The C4b2a complex serves as C3 convertase. In the alternate pathway, C3b serves as the cofactor of the C3-cleaving enzyme complex (C3b, P, Bb), also known as *C3 convertase*. Thus, C3b serves to prime its own activation. The C3b formed through the classic pathway can directly initiate the assembly of the alternate pathway C3 convertase. C3 can also be activated by spontaneous hydrolysis. The C3b complexes formed by the classic and alternate pathways activate C5 to C5a and C5b. Membrane damage is initiated by the assembly of C5b with C6, 7, 8, 9.

macrophages. C3c dissociates from the membrane, but C3d remains attached. Erythrocytes coated with C3d have a normal survival because macrophages have no receptors for this complement component. Thus, the balance between C3b deposition on the membrane and C3b inactivation determines the susceptibility of erythrocytes to phagocytosis by macrophages via the C3b receptors.

In addition to C3b inactivator, other inhibitors of complement activation exist. Web Table 19-1 lists some of these inactivators and their functions.

IgM-Mediated Hemolysis

In cold agglutinin syndrome, IgM molecules attach to the erythrocyte membrane, but these sensitized cells are not removed from circulation in the same manner as those sensitized with IgG because macrophages do not have receptors for the Fc portion of IgM. However, IgM is an efficient activator of complement, and cells can be lysed intravascularly if complement activation through C9 is complete. If activation is incomplete and only C3b coats the erythrocytes, they can be destroyed extravascularly via adherence to CR1 and CR3 receptors on macrophages. Adherence of the cell to macrophages via complement receptors and subsequent phagocytosis, however, is less efficient than immune adherence (adherence mediated via immunoglobulin) and phagocytosis via macrophage Fcγ receptors. It has been estimated that $>$100,000 molecules of the complement component C3b are required on the cell surface to induce effective macrophage phagocytosis via complement alone. C3b is also inefficient in promoting adherence to macrophages because much of the C3b is inactivated to C3d. Thus, extravascular hemolysis of cells sensitized with complement is not as severe as hemolysis of cells sensitized with IgG.

In addition to activating complement, IgM antibodies agglutinate cells. In vitro, agglutination is a useful phenomenon for detecting the presence of cold agglutinins.

☑ CHECKPOINT 19-3

Compare the mechanisms of IgG-mediated hemolysis with those of IgM-mediated hemolysis.

LABORATORY IDENTIFICATION OF SENSITIZED RED CELLS

Suspicion of immune hemolytic anemia indicates specific tests to detect and identify the causative antibody. In general, two distinct techniques are used:

- Agglutination in saline, which will detect IgM antibodies
- **Antihuman globulin (AHG)** test, which will detect IgG antibodies and/or complement

IgM antibodies can be detected by agglutination reactions using test sera (antibody) and appropriate erythrocytes suspended in saline, but IgG antibodies cannot. The difference in the ability of IgG and IgM to cause agglutination in saline can be explained by the difference in size of the two antibodies in relation to the zeta potential. The erythrocyte zeta potential is an electrostatic potential created by the ionic cloud surrounding erythrocytes when they are suspended in saline. This force tends to keep the erythrocytes about 25 nm apart in solution. Thus, an antibody must be large enough to span the 25 nm

gap between cells in order to agglutinate saline- or plasma-suspended cells. The IgM pentamer has a possible span of 35 nm; therefore, it can overcome the electrostatic forces separating the cells and cause agglutination (Figure 19-3 ■). However, the maximum span of an IgG molecule is about 14 nm, and it cannot bridge antigens on two separate cells to cause agglutination. Thus, detection of IgG antibodies requires a different technique using AHG, which is an antibody to human IgG. AHG can connect the antibody molecules on separate cells and cause agglutination.

The AHG test (historically referred to as the *Coombs test*) is a laboratory procedure designed to detect erythrocytes sensitized with IgG and/or complement. Polyspecific AHG is composed of antibodies to both IgG and C3, capable of attaching to the Fc region of IgG immunoglobulins or complement components on two separate cells, bridging the distance between them and leading to the lattice formation known as *agglutination*. Monoclonal antiserum against IgG only (anti-IgG AHG) or C3 only (anti-C3 AHG) are used to specifically identify what component is on the erythrocyte.

The two types of tests using AHG are:

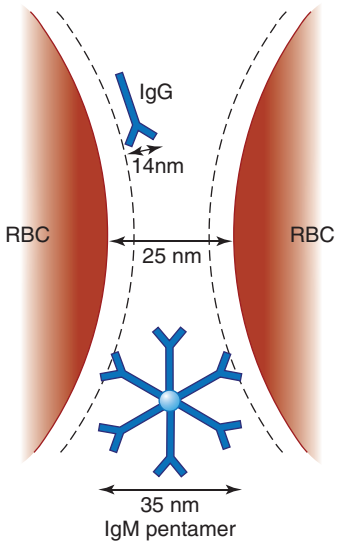
- Direct antiglobulin test detects erythrocytes coated with antibody or complement in vivo
- Indirect antiglobulin test detects antibodies in the plasma or serum

Direct Antiglobulin Test

The **direct antiglobulin test (DAT)** detects erythrocytes that have been sensitized with antibody and/or complement in vivo (Web Figure 19-1). This test should always be performed in suspected cases of AIHA because it differentiates AIHA from all other types of hemolytic anemia, confirming the immunologic basis of erythrocyte destruction. Specimens collected in tubes with ethylenediaminetetraacetate (EDTA) are preferred to clotted specimens for the DAT procedure. EDTA chelates Ca^{++} and Mg^{++}, preventing the in vitro binding of complement to red cells that can be mediated by naturally occurring cold-reactive antibodies (e.g., autoanti-I). However, complement that has been bound in vivo will be detected. Polyspecific AHG is added to saline-washed patient cells, and agglutination is considered positive evidence for the presence of IgG and/or complement components on the cells due to in vivo coating.

A positive test with polyspecific AHG should be followed by a DAT with monospecific AHG antiserum that reacts specifically with either IgG or complement to determine the type of proteins bound to the erythrocyte. Either or both of the monospecific IgG-AHG and C3b-AHG can be positive. If the anti-IgG test is positive, the antibody can be removed from the cell by an elution process, and the resulting eluate (solution containing the antibody) tested to identify the specificity of the antibody.

If an autoantibody is IgM, only complement usually is detected on the erythrocytes as the IgM tends to dissociate from the cells in the warmer part of the circulation. The polyspecific and the anti-C3 monospecific DAT test will be positive; the anti-IgG will be negative. An elution procedure is not used if only complement is detected on the erythrocyte because no antibody would be recovered for identification. In general, if the antibody coating the cells is IgA, the DAT will be negative because the AHG reagent cannot detect that immunoglobulin class.



■ **FIGURE 19-3** The zeta potential of erythrocytes keeps the cells about 25 nm apart when suspended in saline. IgG antibodies have a span of about 14 nm, not enough to bridge the gap between cells and cause agglutination. IgM antibodies, however, are pentamers with a span of about 35 nm, a distance sufficient to bridge the space between cells and cause agglutination.

Indirect Antiglobulin Test

The **indirect antiglobulin test (IAT)**, sometimes referred to as an *antibody screen*, is used to detect antibodies in the patient's serum. A positive IAT indicates alloimmunization (immunization to antigens from another individual) and/or the presence of free autoantibody in the patient's serum. In the IAT, free antibody is detected by incubating the patient's serum with reagent erythrocytes of known antigenic makeup (commonly referred to as *screening cells*). An autocontrol consisting of the patient's serum and erythrocytes is also performed. After a specified incubation period, the cells are washed free of excess serum, and AHG antiserum is added. If the patient's antibody has attached to the corresponding erythrocyte antigen during the incubation period, the reagent cells will agglutinate with AHG (Web Figure 19-2). Further testing to identify the specificity of the antibody is then carried out.

☑ CHECKPOINT 19-4

Compare the purpose of the DAT and the IAT, and state the type of specimen used for each test.

Negative DAT in AIHA

In a few cases of AIHA, antibody cannot be detected on the patient's cells or in the serum.[13,14] This can result from an insufficient number of IgG molecules on the erythrocyte for detection, autoantibodies of

the IgA or IgM class, or the presence of autoantibodies with a low affinity for the erythrocyte.[1,15,16] The DAT can detect as few as 100–500 molecules of IgG per cell or 400–1100 molecules of complement per cell.[2,3] However, in vivo removal of sensitized cells by macrophages can occur when cells are coated with fewer IgG molecules. Thus, the in vivo life span of the sensitized cell can be significantly shortened, as evidenced by the clinical findings of a typical hemolytic anemia, but the concentration of antibodies on the cell could be insufficient to give a positive DAT. Newer, more sensitive techniques, such as enzyme-linked DAT, gel tests, dual DAT procedure, Polybrene tests, and flow cytometry can detect lower concentrations of antibodies than the conventional DAT technique.[17–19] IgA or IgM autoantibodies are not detected using polyspecific AHG. However, if complement has been activated, it can be detected if sufficient molecules are present. Specialized antihuman globulin reagents with anti-IgA or anti-IgM specificity is used in some reference laboratories to detect erythrocyte sensitization by immunoglobulins of these classes.

Positive DAT in Normal Individuals

Some healthy blood donors and hospitalized patients can have a positive DAT but not shortened erythrocyte survival. The reason for this observation is not clear,[1] but factors that could be responsible include the following:

1. The individual's macrophages may not be as active in removing sensitized cells as the macrophages in individuals with hemolytic disease.
2. The amount of antibody bound to cells might not be sufficient to cause decreased erythrocyte life span.
3. Macrophages might not recognize the subclass of antibody sensitizing the cell. Macrophage Fc receptors have low affinity for the IgG2 and IgG4 subclasses. Erythrocytes coated with these immunoglobulins will give a positive direct DAT, but in vivo, survival of the cells will be normal.
4. The thermal amplitude of the antibody may be $<37°C$.
5. The positive DAT can be due to the presence of certain complement fragments on erythrocytes. Increased amounts of C3d can be found on the erythrocytes, but because this component is not detected by macrophage receptors, erythrocytes will not have decreased survival.
6. Patients with hypergammaglobulinemia or receiving high-dose intravenous immunoglobulin (IVIG) could have a positive DAT because of nonspecific binding of immunoglobulins to the erythrocytes.

CASE STUDY *(continued from page 351)*

When examining the Nancy's peripheral blood smear, the laboratory professional noted that spherocytes were present. The reticulocyte count was elevated. The laboratory professional called the blood bank and found that the DAT on Nancy was positive with polyspecific AHG, anti-IgG but negative with anticomplement.

2. What is the significance of the spherocytes?
3. Based on these results what do you suspect is going on in Nancy's? Explain.

AUTOIMMUNE HEMOLYTIC ANEMIAS (AIHA)

Usually a person's immune system recognizes self-antigens (those that appear on the individual's own cells) and does not mount an immune response to these antigens. These antigens can, however, stimulate an immune response if injected into another individual because the recipient's immune system recognizes them as being "foreign." The immune regulatory mechanisms that govern response to self-antigens are collectively known as **immune tolerance**.

It is generally accepted that autoimmune diseases occur as the result of a number of factors including genetic predisposition, exposure to infectious agents that can induce antibody production due to molecular mimicry, and defects in the mechanism regulating immune tolerance including altered levels of CR1, lack of effective T regulator cells (T_{Reg}) that maintain peripheral tolerance, and polyclonal lymphocyte activation.[20] T_{Reg} lymphocytes normally induce immune tolerance by inhibiting antibody-producing activity of B lymphocytes to self-antigens. Loss of this suppressor activity could result in the formation of antibodies against self. Loss of self-tolerance and resulting AIHA can occur at any age. The mechanism of antibody formation in AIHA is unknown, but because many AIHA cases are associated with microbial infection, neoplasia, or drug administration, these agents can be involved in alternating self-antigens and subsequent immune system response. Warm or cold AIHA is also categorized based on whether there is an underlying disease associated with it. The two categories are:

- Primary or idiopathic: no underlying disease identified
- Secondary: underlying disease present

Warm Autoimmune Hemolytic Anemia

Warm autoimmune hemolytic anemia (WAIHA) is the most common form of AIHA (up to 70% of cases).[2] It is most often mediated by IgG antibodies (usually IgG1 or IgG3) whose maximal reactivity is at 37°C. In a majority (90%) of WAIHA cases, erythrocytes are sensitized with IgG and complement or IgG alone. Only 7% of cases are sensitized with complement alone. WAIHA is rarely associated with IgM-only or IgA-only sensitization.[4,21]

Although WAIHA can occur at any age, the incidence increases after age 40. Childhood incidence peaks in the first 4 years of life.[22] About 60% of the cases of WAIHA are idiopathic and can be acute or chronic.[2] In acute idiopathic WAIHA, severe anemia develops over 2 to 3 days, but the hemolysis is self-limited with a duration of several weeks to several years. In other instances, the hemolysis is chronic and does not abate.

The underlying disorders most frequently associated with secondary WAIHA are:

- Lymphoproliferative disease, including chronic lymphocytic leukemia (CLL) and Hodgkin's disease (Chapter 28). CLL, found most

frequently in older adults, is the disease most frequently associated with the development of secondary WAIHA.[23] WAIHA often develops late in the disease when the immune dysregulation is greatest. In one study, >60% of the cases of WAIHA were in patients with CLL.[23,24] Many children with idiopathic WAIHA eventually develop a lymphoproliferative disease.

- Neoplastic diseases
- Other autoimmune disorders, including systemic lupus erythematosus and rheumatoid arthritis, ulcerative colitis, and Crohn's disease[25–28]
- Certain viral and bacterial infections[29–31]
- Vaccinations[32,33]
- Solid organ transplant[34,35]

Presence of the underlying disorder can complicate initial diagnosis and treatment of hemolytic anemia. In many cases, the AIHA is resolved by treating the underlying disease.

Pathophysiology

The autoantibody in WAIHA is reactive with antigens on the patient's erythrocytes. Most often the specificity of the antibody is directed against antigens of the Rh system, although other antigen systems can be involved. When directed against the Rh system, the antibody can be specific for a single antigen (such as autoanti-e) or more commonly, the antibody reacts with a complex Rh antigen found on all erythrocytes except Rh null or Rh-deleted cells.[1,2,36] The epitope against which these latter antibodies are directed has not been defined. Recent research suggests that the antibody can be against a component of the erythrocyte membrane such as glycophorins, band 3 protein, or protein band 4.1.[1,37]

Most hemolysis in WAIHA is extravascular via splenic macrophages. Although complement is not needed for cell destruction, if both antibody and complement are on the cell membrane, phagocytosis is enhanced. If erythrocyte destruction exceeds the compensatory capacity of the bone marrow to produce new cells, anemia develops. Direct complement-mediated intravascular hemolysis associated with IgM antibodies in WAIHA is rare.

Clinical Findings

The most common presenting symptoms in idiopathic WAIHA are related to anemia. Progressive weakness, dizziness, dyspnea on exertion, back or abdominal pain, and jaundice are common. In secondary WAIHA, signs and symptoms of the underlying disorder can obscure the features of the hemolytic anemia. The patient can present with signs and symptoms of both the underlying disease and hemolysis or just the disease. The signs of WAIHA in a few patients can precede development of the underlying disease. Although mild to moderate splenomegaly is present in >50% of patients with WAIHA, massive splenic enlargement suggests an underlying lymphoproliferative disorder. Hepatomegaly is found in about one-third of patients with primary WAIHA.

Laboratory Findings

Refer to Table 19-4 ★ for the most common laboratory findings associated with WAIHA. Findings such as a positive DAT, autoantibody in the serum, and presence of spherocytes on the peripheral blood smear reflect the immune-mediated destruction of the erythrocyte.

Peripheral Blood

Moderate to severe normocytic, normochromic anemia is typical. In well-compensated hemolytic disease, anemia can be mild or absent, and the only abnormal parameters can be a positive DAT and an increase in reticulocytes. Reticulocytes are invariably increased in uncomplicated hemolytic disease. The reticulocyte production index can be as high as 6 or 7. Depending on the degree of reticulocytosis, macrocytosis can be present.

The blood smear frequently shows erythrocyte abnormalities that suggest a hemolytic process. Polychromasia, nucleated red blood cells, spherocytes, schistocytes, and other poikilocytes are characteristic (Figure 19-4 ■). Spherocytes found in AIHA are usually more heterogeneous than are the spherocytes associated with hereditary spherocytosis. This anisocytosis is readily noted upon examining the blood smear and is indicated by an increase in red cell distribution width (RDW) on automated hematology analyzers. Erythrophagocytosis by monocytes is rarely seen. The monocyte-engulfed erythrocyte is readily detected if the cell still contains its pink-staining hemoglobin. If the hemoglobin has leaked out of the cell, however, only colorless vacuoles are seen. Leukocyte counts are normal or increased with

★ **TABLE 19-4** Laboratory Findings Associated with WAIHA

Common findings	• Positive DAT • Normocytic, normochromic anemia • Increased reticulocytes • Spherocytes and other erythrocyte abnormalities • Presence of autoantibody in the serum • Increased serum bilirubin (total and unconjugated) • Decreased serum haptoglobin • Elevated lactate dehydrogenase (LD) • Positive antibody screen with all cells including autocontrol • Incompatible crossmatches with all donors
Other laboratory findings that can be associated with hemolysis in WAIHA	• Increased osmotic fragility • Increased urine and fecal urobilinogen • Hemoglobinemia,[a] hemoglobinuria, methemoglobinemia, hemosiderinuria

[a]Seen only in acute hemolytic episode

☑ CHECKPOINT 19-5

What are the clinical findings and the immune stimuli for WAIHA?

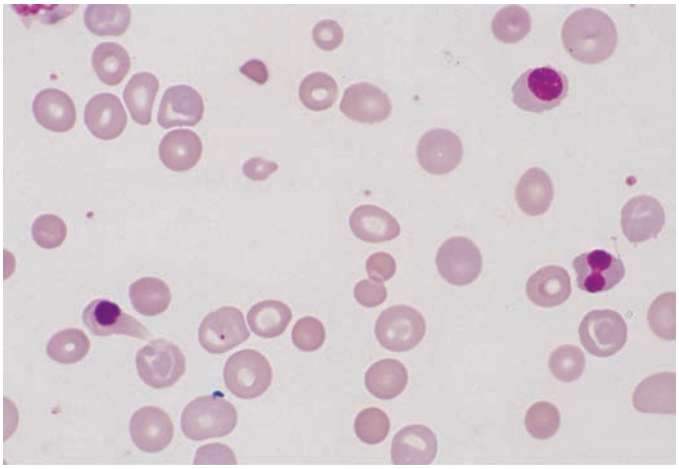


FIGURE 19-4 A blood smear from a patient with warm autoimmune hemolytic anemia (WAIHA). The marked anisocytosis is due to the presence of spherocytes and large polychromatophilic erythrocytes. The nucleated cells are erythroblasts (Wright stain, 1000× original magnification).

neutrophilia in idiopathic WAIHA but can vary in the secondary form based on the underlying disease. Platelet counts are usually normal or slightly increased. When severe thrombocytopenia accompanies WAIHA, the disease is called *Evan's syndrome*.

Bone Marrow

Bone marrow examination is not necessary for the diagnosis of WAIHA. The bone marrow can show erythroid hyperplasia, depending on the degree of hemolysis. Erythrophagocytosis by macrophages can be seen. Compensatory bone marrow response can be less than expected with concomitant folic acid deficiency. In chronic hemolysis, the folic acid requirement increases two to three times normal; without folic acid supplements, the stores are quickly depleted. If the patient contracts viral infections associated with bone marrow suppression, life-threatening anemia (aplastic crisis) can occur.

Other Laboratory Tests

The DAT is a useful test to distinguish the immune nature of this hemolytic anemia from nonimmune-mediated hemolytic anemias. The test is usually positive with polyspecific AHG and anti-IgG monospecific AHG antiserum. Only about 30% of the cases of WAIHA have a reaction with anti-C3, either with or without the concurrent presence of IgG.[2]

Reaction of patients' serum with commercial screening cells typically shows agglutination with all cells when AHG is added. The autocontrol (patient serum and patient erythrocytes) shows similar reactions. Other laboratory findings are nonspecific but reflect the hemolytic component of the condition (Table 19-4).

Differential Diagnosis

WAIHA with the presence of spherocytes can be differentiated from hereditary spherocytosis (HS) by the DAT. Antibodies are not responsible for forming spherocytes in HS; therefore, the DAT is negative. The autohemolysis test is abnormal in both HS and in WAIHA with spherocytes. The addition of glucose significantly corrects autohemolysis in HS, but not in WAIHA. The peripheral blood smear also gives clues to diagnosis. The spherocytes in HS appear as a rather homogeneous population, but WAIHA has a mixture of spherocytes, microspherocytes, and normocytes.

☑ CHECKPOINT 19-6

What is the DAT pattern in WAIHA? Explain why spherocytes are commonly seen in WAIHA.

Therapy

Transfusion therapy is not necessary in self-limiting hemolytic disorders without life-threatening anemia. When transfusion is indicated (more often associated with acute onset), finding a suitable donor is difficult because the patient's autoantibody usually reacts with all donor cells in the cross-match. In addition, approximately 30% of patients with WAIHA have underlying alloantibodies, which can present an additional challenge to finding compatible blood because the autoantibody can mask their presence.[37–39]

If the patient has an alloantibody, blood negative for the specific antigen must be administered. If serologically compatible blood cannot be found because of the presence of the autoantibody, donor cells demonstrating the least incompatibility are usually chosen. In some cases, the blood transfused is matched only for ABO and Rh type.[39] The clinical problems with this approach, however, are twofold: Donor cells (1) are often destroyed as rapidly as the patient's own erythrocytes and (2) can also stimulate the production of alloantibodies. In some cases, the use of flow cytometry can help evaluate the survival of donor cells in patients with WAIHA. In other cases, such as individuals who must receive serial transfusions, finding phenotypically matched donor blood can decrease the risk of stimulating alloantibodies.[38–41] If the autoantibody has an identifiable single specificity (such as autoanti-e), the donor blood chosen for the compatibility test should be negative for the antigen.

Therapies for WAIHA focus on decreasing the production of autoantibodies and slowing down the destruction of erythrocytes. These therapies include:

- **Corticosteroids** The standard initial or first-line therapy for patients with WAIHA is often a course of immunosuppressive drugs such as corticosteroids. Corticosteroids are used to produce immunosuppression by decreasing lymphocyte proliferation and suppressing macrophage sequestration of sensitized cells by affecting the Fc receptors. Although many patients show a good initial response with a decrease in erythrocyte destruction, few patients experience complete remission with this therapy. A decrease in production of autoantibodies is often demonstrated by a decrease in the strength of the DAT reaction and indirect antiglobulin test. Response usually occurs within 10–14 days, and many patients require long-term low-dose maintenance.[41,42]

- **Danazol** This drug is an attenuated androgen that can be used as a first-line treatment with corticosteroids.[37,41]

Standard second-line treatments with the most efficacy for individuals who do not respond to corticosteroids includes spelenctomy or the use of monoclonal antibody treatment (rituximab).[37,39,41]

- **Splenectomy** Splenectomy provides long-term remission for some patients. Removal of the spleen decreases the destruction of IgG-coated erythrocytes that would normally be removed by splenic macrophages. If, however, the antibody concentration remains high, the destruction of sensitized erythrocytes can continue in the liver. Some evidence suggests that splenectomy can be more beneficial in patients with idiopathic WAIHA than in those with the secondary type.[41] The primary complication is sepsis related to infections, especially with encapsulated bacteria.
- **Rituximab (monoclonal anti-CD20)** Rituximab has been used in the treatment of clonal B-cell malignancies, including chronic lymphocytic leukemia, in cases of autoimmune collagen vascular disease and in patients with idiopathic and secondary WAIHA that are refractory to other treatments.[41,43] It functions by depleting the B cells that produce autoantibodies.[37,41]
- **Cytotoxic drugs** Various alternative therapies are available for patients who do not respond to first- or second-line therapy or who have recurrent disease.[1,41] Cytotoxic drugs such as cyclophosphamide, cyclosporine A, vincristine-loaded platelets, mycophenolate mofetil, and azathioprine are used to cause general suppression of the immune system, which decreases synthesis of autoantibody.[1,37]
- **High-dose intravenous immunoglobulin (IVIG)** This treatment blocks Fc receptors on macrophages and affects T- and B-cell function by increasing T_{Reg} cells or reducing B-cell function. It has a variable success rate and is most often used as an adjunct therapy with corticosteroids or in selected patients with severe anemia who are refractory to drugs.[42]
- **Therapeutic plasma exchange and plasmapheresis** This procedure can dilute or temporarily remove the autoantibody from the patient's circulation. It has been successful in reducing antibody load for a short time in some cases but is not a satisfactory long-term therapy.[41]

Other treatments such as stem cell transplant have not proven to be effective.[39,41] Treatment of the underlying disease in patients with secondary WAIHA is important. Often resolution of the disease leads to decreased production of the autoantibody.

Cold Autoimmune Hemolytic Anemia

Cold AIHA, also termed *cold agglutinin disease (CAD)* or **cold agglutinin syndrome (CAS)**, is associated with an IgM antibody that fixes complement and is reactive below 37°C. This disorder, which comprises 16–30% of the AIHAs, is less common in children than adults.[1,2,44,45] IgA and IgG antibodies rarely have been implicated in hemolysis in CAS.[44–46]

CAS, like WAIHA, is either idiopathic or secondary (Table 19-5 ★). Idiopathic CAS is usually chronic, occurring after age 50 with a peak onset after age 70.[21] The antibody involved is usually a monoclonal IgM/κ light chain with autoanti-I specificity.[45,47] The secondary type can occur after an infectious disease or be associated with lymphoproliferative disease.[21,44–47] The secondary type associated with infectious disease is usually an acute, self-limiting form that has an onset 1–3 weeks after infection and resolves within 2–3 weeks.[44,45] Most of these autoantibodies are polyclonal and have a specificity for antigens of the Ii system. Anti-I is usually associated with *Mycoplasma pneumoniae* infections and anti-i can be seen in infectious mononucleosis. Infectious agents including varicella (chickenpox) and rubella are associated with anti-Pr but rarely with anti-I specificity.[48,49] HIV patients can also develop anti-I.[50] The secondary chronic form of CAS associated with lymphoproliferative disorders, such as lymphoma or Waldenstrom's macroglobulinemia, is typically found in older individuals.[46] The antibody in these cases is usually a monoclonal IgMκ protein. A more severe type of cold AIHA, paroxysmal cold hemoglobinuria (PCH), is associated with a biphasic cold-reacting IgG antibody and is discussed in the next section.

★ **TABLE 19-5** Autoimmune Hemolytic Anemia Caused by Cold-Reacting Antibodies

Cold agglutinin syndrome (CAS)	Primary (idiopathic) Secondary • Viral and bacterial infections • *Mycoplasma pneumonia* infections, infectious mononucleosis • Lymphoproliferative diseases
Paroxysmal cold hemoglobinuria	Primary (idiopathic) Secondary • Viral infections • Tertiary syphilis

Pathophysiology

The severity of CAS is related to the thermal range of the antibody. Cold-reacting antibodies with a wide range of thermal activity (up to 32°C) can cause problems when the peripheral circulation cools to this temperature. Complement-mediated lysis accounts for most of the erythrocyte destruction.

The cold-reacting antibody is usually directed against the I antigen, which is expressed on erythrocytes of almost all adults. I antigen specificity of the antibody can be defined by reactivity of the patient's serum with all adult erythrocytes but minimal or no reactivity with cord cells (which lack the I antigen). In CAS associated with infectious mononucleosis and lymphoproliferative disease, however, the antibody may have anti-i specificity.[31] These antibodies react strongly with cord cells because the i antigen is generally expressed strongly on cord cells and erythrocytes of children younger than 2 years old and weakly or not at all with adult erythrocytes. The second most common specificity for cold autogglutinins is anti-Pr.[1] The Pr antigens are expressed on both adult and infant erythrocytes.

Clinical Findings

In some instances, CAS is associated with a chronic hemolytic anemia with or without jaundice. In others, hemolysis is episodic and associated only with chilling. Erythrocyte agglutination occurs in areas of the body that cool to the thermal range of the antibody and cause sludging of the blood flow within capillaries. Vascular changes including acrocyanosis in which the hands and/or feet turn blue and cold can occur. In *Raynaud's phenomenon*, pain can be accompanied by a characteristic pattern of color changes in the skin from white (due to spasm of the vessels) to blue (caused by cyanosis) to red (which indicates a return of blood flow to the area). These conditions primarily affect the extremities, especially the tip of the nose, fingers, toes, and ears. Hemoglobinuria accompanies the acute hemolytic attacks. Splenomegaly can be present (Table 19-6 ★).

☑ CHECKPOINT 19-7

Describe the mechanism of cell destruction in CAS.

Laboratory Findings

The first indication of the presence of unsuspected cold agglutinins often is from blood counts performed on electronic cell counters. The erythrocyte count is inappropriately decreased for the hemoglobin content. and the mean cell volume (MCV) is falsely elevated (Table 19-6). These erroneous values occur when erythrocyte agglutinates are sized and counted as single cells. The hematocrit, calculated from the erroneous erythrocyte count and MCV, is falsely low. The mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) calculated from the erythrocyte count and hematocrit are falsely elevated. The hemoglobin assay is accurate because the cells are lysed to determine this parameter. Accurate cell counts can be obtained by warming the blood and diluting reagents to 37°C before performing the test. Visible autoagglutination in tubes of anticoagulated blood can be observed as the blood cools to room temperature.

When blood counts are performed at 37°C, the results indicate a mild to moderate normocytic, normochromic anemia. The blood film shows polychromasia, some spherocytes, rouleaux, or clumps of erythrocytes and sometimes nucleated red cells (Figure 19-5 ■). Erythrophagocytosis can be seen but is more typical on smears made from buffy coats after the blood has incubated at room temperature. Leukocyte and platelet counts are usually normal. Leukocytosis can occur during acute hemolysis as the result of a bone marrow stress response. The bone marrow exhibits normoblastic hyperplasia often with an increase in lymphoid cells. Patients with chronic CAS can show decreased C3 and/or C4 levels.[47] Patients also can have a decreased value for the CH50 assay, a functional hemolytic assay that measures the integrity of the entire complement cascade.

★ **TABLE 19-6** Criteria for Clinical Diagnosis of Cold Agglutinin Syndrome (CAS)

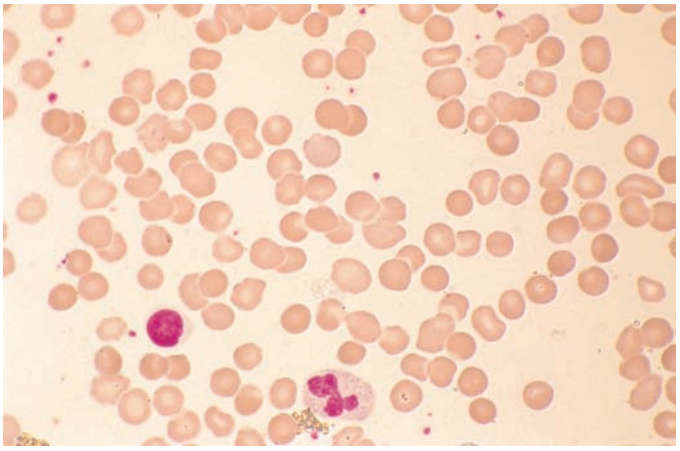
Clinical history	• Acrocyanosis • Hemoglobinuria on exposure to cold
Laboratory findings	• Serological DAT: Positive with polyspecific AHG Negative with anti-IgG; Positive with anti-C3 IAT: Antibody showing characteristic reactions at <25°C Cold agglutinin titer >1000 at 4°C • False increase in MCV, MCH, and MCHC • False decrease in erythrocyte count • Normocytic, normochromic anemia • Reticulocytosis • Spherocytes, agglutinated RBCs, rouleaux, nucleated RBCs on blood smear • Increased bilirubin (total and unconjugated) • Decreased haptoglobin • Hemoglobinemia, hemoglobinuria in acute hemolysis • Hemosiderinuria in chronic hemolysis

☑ CHECKPOINT 19-8

Explain why the MCV, MCH, and MCHC can be falsely increased when blood from someone with CAS is tested using an automated cell counter.

Differentiation of CAS Agglutinins from Benign Cold Agglutinins

The serum of most normal individuals exhibits the presence of cold autoantibodies when the serum and cells are incubated at 4°C. These antibodies are termed *benign cold autoagglutinins* because their



■ **FIGURE 19-5** Cold autoimmune hemolytic anemia from a patient with chronic lymphocytic leukemia. Some of the erythrocytes are in small clumps. Spherocytes are also present (peripheral blood, Wright stain, 1000× original magnification).

thermal amplitude and concentration (titer) are not high enough to cause clinical problems. When pathologic cold agglutinins are suspected as the cause of anemia, laboratory tests should be performed to differentiate pathologic cold agglutinins from the benign ones (Table 19-7 ★). The DAT with polyspecific AHG and monospecific anticomplement antiserum is positive in pathologic cold agglutinin disease but negative or only weakly positive with benign cold agglutinins. The DAT, utilizing monospecific anti-IgG, is negative both in CAS and benign cold agglutinins.

Historically, the cold agglutinin test was performed when CAS was suspected. This test demonstrates the ability of the pathologic antibody to agglutinate the patient's cells at temperatures from 0°C to 20°C in saline. The reaction is reversible with agglutinates dispersing at 37°C. With benign cold antibodies, agglutination occurs at 0–4°C and can occur up to 20°C. Titers of benign cold agglutinins reach 1:64 in normal individuals; in cold agglutinin disease, the titer is usually >1:1000. Titers of 1:256 or more with a positive DAT using monospecific anti-C3 antisera and a negative DAT using monospecific anti-IgG antisera are highly suggestive of cold agglutinin disease.

☑ CHECKPOINT 19-9

Compare the DAT findings and the antibody specificity in WAIHA and CAS.

Therapy

The most effective therapy in primary CAS is usually achieved by keeping the individual's extremities warm. Corticosteroids are not usually effective. A few cases have responded to danazol.[44] Chemotherapy using rituximab has been somewhat effective in primary CAS and CAS associated with an underlying lymphoproliferative disease.[39,44,51–53] Plasma exchange can be used in acute hemolytic episodes because the majority of IgM is distributed in the intravascular spaces. However, plasma exchange is effective for only a short time because protein regeneration half-life is ~5 days.[14,50] Splenectomy, which can be effective in WAIHA, is not effective in CAS because the C3b-coated cells are destroyed primarily by Kupffer cells in the liver. Patients with strongly reacting cold agglutinins can have complications if surgical procedures requiring hypothermia are undertaken.[46,50]

Paroxysmal Cold Hemoglobinuria

Paroxysmal cold hemoglobinuria (PCH) is a rare autoimmune hemolytic disorder that can occur at any age and is characterized by massive intermittent acute hemolysis and hemoglobinuria. Although it is the cause of about 2% of autoimmune hemolytic anemias overall, it is the cause of 30–40% of AIHA in children and is most frequent in children under the age of 5 years.[1,22,54] Historically, PCH was associated with congenital or tertiary syphilis in adults. It is now most often seen in children with viral and bacterial infections although it has been reported in association with some lymphoproliferative diseases in adults.[55] The infections linked to PCH include Epstein-Barr virus, cytomegalovirus, measles, mumps, *Haemophilus influenzae*, and *Klebsiella pneumoniae*.[1,56] Parvovirus 19, which has an affinity for the P antigen, has also been linked to PCH.[57] PCH is usually a transient disorder that appears 5 days to 3 weeks after infection onset but can persist up to 3 months.[44] It generally resolves spontaneously, but transfusion can be required in cases of severe, life-threatening anemia.

Pathophysiology

PCH was the first hemolytic anemia for which a mechanism of hemolysis was established. This hemolytic anemia is distinct from the other cold AIHAs because of the nature of the antibody involved. It is caused by a biphasic complement-fixing IgG antibody, the **Donath-Landsteiner (D-L) antibody**. *Biphasic* refers to the two temperatures necessary for optimal lysis of the erythrocytes. The antibody reacts with erythrocytes in the capillaries at temperatures <20°C and avidly binds the early acting complement components. Upon warming to 37°C, the antibody molecule detaches from the cell, but the membrane attack complement components are activated on the cell membrane causing cell lysis. Because the antibody repeatedly attaches and detaches from erythrocytes with subsequent complement activation, it can cause significant hemolysis.[58] The PCH antibody is specific for the P-antigen (autoanti-P).

Clinical Findings

Hemoglobinuria is the most common clinical symptom. The patient can also experience jaundice, pallor, and hepatosplenomegaly. Raynaud's phenomenon can occur during acute episodes followed by jaundice.

★ **TABLE 19-7** Comparison of Characteristics of Pathologic Cold Agglutinins Found in CAS with Those of Benign Cold Agglutinins Found in Normal Individuals

	Pathologic Agglutinins	Benign Agglutinins
Antibody class	IgM	IgM
Antibody specificity	Usually anti-I but in secondary CAS can be anti-i	anti-I
Antibody clonality	Monoclonal in idiopathic type and secondary type due to lymphoproliferative disease; polyclonal in secondary type due to infectious disease	Polyclonal
Thermal amplitude	0–30°C	0–4°C
Agglutination at room temperature	Significant	Not present
Titer	Usually >1:1000	<1:64
DAT	Positive with polyspecific AHG and monospecific anticomplement	Negative

Laboratory Findings

The degree of anemia depends on the frequency and severity of hemolytic attacks. During the attack, hemoglobin concentration drops sharply accompanied by hemoglobinemia, methemalbuminemia, and hemoglobinuria. Hemoglobin values can decrease to as low as 5 g/dL. Neutropenia, a neutrophilic shift to the left, reticulocytopenia, and spherocytes can accompany erythrocyte lysis. Serum bilirubin, blood urea nitrogen, and lactic dehydrogenase (LD) are elevated, whereas serum complement and haptoglobin are decreased. Erythrophagocytosis occurs more commonly in PCH than in other types of AIHA, and the phagocytic cells usually involved are segmented neutrophils, not monocytes.[59]

Antibodies on the cells are not usually detected by the DAT because the D-L antibody elutes at warm temperatures. A weakly positive DAT with anticomplement AHG can appear and persist for several days after the hemolytic episode. The IAT can be positive if performed in the cold. Normal erythrocytes incubated with patient serum react more positively in the IAT than will patient cells.

D-L antibodies are usually present in low titers (<1:32) and express a low thermal amplitude, but their presence can be verified by the D-L test, which employs a biphasic reaction (Table 19-8 ★). In this test, the patient's blood is collected in two clot tubes (serum, not plasma, is required); one is incubated at 4°C for 30 minutes and the other at 37°C for 30 minutes. Both tubes are then incubated at 37°C. If the D-L antibody is present, it causes hemolysis in the tube initially incubated at 4°C and then warmed to 37°C. No hemolysis is present in the tube kept at 37°C. Hemolysis in this test can also occur in cold agglutinin syndrome (CAS), but the hemolysis occurs very slowly. See Table 19-9 ★ for a comparison of PCH and CAS.

Therapy

PCH associated with acute infections terminates upon recovery from the infection. Steroids are not usually helpful. Transfusion can be required if the hemolysis is severe. In rare cases when the hemolysis persists, plasmapheresis can be used.[58] Rituximab has also been used as therapy in rare adult cases.[60] The chronic form of the disease is best treated by avoiding exposure to the cold.

☑ CHECKPOINT 19-10

Compare the antibody specificity and the confirmatory test for PCH and CAS.

★ **TABLE 19-8** Donath-Landsteiner (D-L) Test for Detecting the Presence of D-L Antibodies[a]

Patient's Whole Blood[a]	Control	Test
Incubate for 30 min at	37°C	4°C
Incubate for 30 min at	37°C	37°C
Centrifuge: observe plasma for presence of hemolysis		
Interpretation		
D-L antibodies present	No hemolysis	Hemolysis
No D-L antibodies present	No hemolysis	No hemolysis

[a]Two tubes of patient's whole blood are used; one tube serves as the control and the other as the test.

Mixed-Type AIHA

Mixed-type AIHA is characterized by the presence of a warm-reacting IgG autoantibody and a cold-reacting IgM autoantibody that has both high titer and increased thermal amplitude (reacts at >30°C). About 50% of the cases are idiopathic; most of the remainder are associated with diseases such as systemic lupus erythematosus, lymphoma, and HIV.[28,50,61] Patients frequently present with an acute, severe anemia and can have a more chronic course with intermittent exacerbations. The IgG antibody mediates extravascular hemolysis, and the IgM is responsible for complement fixation and intravascular hemolysis. In some cases, the cold-reacting antibody triggers increased hemolysis when the patient is exposed to the cold.[62]

Both C3 and IgG can be detected on the erythrocyte in the DAT. The cold-reacting antibody often has specificity for the Ii system antigens. The warm-reacting autoantibody is similar to those found in classic WAIHA and often has a complex Rh specificity.

Patients usually respond well to treatment with corticosteroids and do not require transfusion. Rituximab has been used in cases with underlying lymphoproliferative disease.[51,63]

Drug-Induced Hemolytic Anemias

Although the occurrence is rare, certain drugs can cause immune cytopenias that involve one or more cell lineages including neutrophils, platelets, and erythrocytes. Anemia, thrombocytopenia, and agranulocytosis can occur together or separately. It has been

★ **TABLE 19-9** Comparison of Cold Agglutinin Syndrome (CAS) and Paroxysmal Cold Hemoglobinuria (PCH)

	CAS	PCH
Patient	Usually adults >50 years of age	Usually children after viral infection
Clinical findings	Acrocyanosis	Chills, fever, hemoglobinuria
DAT	Positive with polyspecific AHG and monospecific C3	Positive with polyspecific AHG and monospecific C3
Donath-Landsteiner test	Negative	Positive
Antibody class	IgM	Biphasic IgG (D-L)
Antibody specificity	Anti-I	Anti-P
Thermal amplitude of antibody	Up to 30°C	Under 20°C
Hemolysis	Chronic extravascular/intravascular	Acute intravascular
Therapy	Avoid the cold	Supportive; treatment of underlying illness

proposed that a drug's ability to induce production of antibodies against different cell lineages is related to its affinity for the cells. The greater the affinity, the more likely sensitization against the drug–cell complex is to occur. Drug-induced immune hemolytic anemia is a relatively uncommon acquired condition precipitated by certain drugs. The drug itself does not cause erythrocyte injury, and not all individuals taking the drug develop this immune reaction.

More than 125 drugs have been found to induce a positive DAT or an immune-mediated hemolytic anemia.[64,65] The classes of drugs implicated include antimicrobials, nonsteroidal anti-inflammatory drugs, antineoplastic drugs, diuretics, and antidiabetic drugs. Some of the specific drugs are listed in Web Table 19-2. Note that second- and third-generation cephalosporins such as cefotetan and ceftriaxone constitute the majority of cases of drug-induced immune hemolytic anemia and are responsible for most fatalities with ceftriaxone causing >50% of the fatalities.[66–68] Piperacillin and fludarabine are other commonly implicated drugs.[69,70] Drug-induced immune hemolysis must be distinguished from both drug-induced nonimmune hemolysis that occurs secondarily to erythrocyte metabolic defects such as G6PD deficiency and from spontaneous autoimmune disorders. This is important because drug-induced, immune hemolytic anemias are the result of an immune response to drug-induced alteration of the erythrocyte. The resolution for this immune hemolysis involves withdrawal of the offending drug and supportive treatment, not the use of immunosuppressives or other therapy.

The actual mechanisms of drug-induced hemolytic anemia are controversial and still not well defined. At one time, the following mechanisms were proposed to explain them:[1,2]

- Drug absorption
- Immune complex formation
- Autoantibody production

A fourth mechanism, membrane modification, resulted in changes in the erythrocyte membrane but was not correlated with immune hemolysis.

However, in recent years, a "unifying hypothesis" has been proposed to explain antibody formation in drug-induced hemolytic anemias.[1,2,65,71] The hypothesis proposes that once a drug binds to the erythrocyte membrane, antibodies can be produced to react with epitopes specific to the drug, with epitopes that represent a combination of drug and erythrocyte proteins, or with epitopes primarily on the erythrocyte membrane. This helps to explain how patients can develop more than one type of drug-induced antibody.[71,72]

Currently, two major types of drug-induced antibodies have been described: drug dependent and drug independent. They are named based on the type of in vitro testing required for identification. Drug-dependent antibodies are those that require the presence of the drug during in vitro testing. Drug-independent antibodies are those that react in vitro without the presence of the drug.[64,65]

Mechanism of In Vivo Action for Drug-Dependent Antibodies

The mechanism of action for the drug-dependent type antibodies can occur in two ways. In one, the drug binds covalently to the erythrocyte and antibodies are produced only against the drug epitopes. Antibody forms and attaches to the drug-coated erythrocyte, which is then removed by splenic macrophages. Drugs often associated with this mechanism are IV penicillin, piperacillin, and cephotetan.[2,65] The second mechanism for drug-dependent antibody occurs when the drug binds only weakly to the erythrocyte and antibody is formed to epitopes that consist of both drug and erythrocyte membrane. The antibody is able to fix complement and cause intravascular lysis.[65,73] Ceftriaxone is one of the drugs commonly associated with this mechanism.

Mechanism of In Vivo Action for Drug-Independent Antibodies

In the drug-independent mechanism, the drug binds to the erythrocyte; however, the antibody is produced against primarily erythrocyte epitopes. This can be due to alteration of the erythrocyte membrane by the drug or molecular mimicry. This mechanism mimics warm autoimmune hemolytic anemia in that the patient's serum reacts with all erythrocytes against which it is tested. The mechanism by which antibody production is induced is unknown; however, evidence indicates that the drug alters normal erythrocyte antigens so they are no longer recognized as self.[2,74] Historically this mechanism was linked to the antihypertensive drug Aldomet (α-methyldopa). In approximately 20% of patients on the drug, a positive DAT develops after about 3–6 months. However, hemolytic anemia develops in only 1% of these patients. Fludarabine (a purine nucleoside analog used to treat some cancers) now is the drug most commonly associated with this type of drug-independent mechanism for antibody formation.[2,65] Erythrocyte destruction is extravascular, and anemia develops gradually. If the drug is withdrawn, the antibody production gradually stops, but the DAT can remain positive for years. The DAT is dose dependent: The larger the dose of the drug, the more likely the patient is to have a positive DAT. The DAT using anti-IgG is positive but because complement is rarely activated, the DAT using anti-C3 is usually negative.

A second drug-independent mechanism called nonimmunological protein adsorption (NIPA) has been proposed. Some β-lactamase inhibitors, cefotetan, and platinum-based chemotherapeutic agents can alter the erythrocyte membrane so that immunoglobulins and other serum proteins bind nonspecifically to the membrane.[64,70,73] No drug antibody is involved. This mechanism is known to cause hemolysis in some patients. The DAT using polyspecific AHG is positive; the anti-IgG or anti-C3 can be positive or negative.

Table 19-10 ★ contains a summary of the characteristics of each mechanism. Regardless of the underlying mechanism, erythrocytes sensitized with either antibody and/or complement have a shortened life span. Each of these mechanisms causes a positive DAT with polyspecific AHG and with either anti-IgG and/or anti-C3 AHG.

☑ CHECKPOINT 19-11

Compare the different types of drug-induced hemolysis including the type of hemolysis, the drug usually associated with the mechanism, and the DAT profile.

★ **TABLE 19-10** Summary of Classic Mechanisms of Drug-Induced Immune Hemolytic Anemia

Type (Prototypic Drug)	Action	Direct Antiglobulin Test	Mechanism of Cell Destruction
Drug dependent	Drug bound to cell → antibody forms primarily to drug epitopes and binds to drug	Polyspecific AHG positive, anti-IgG positive, anti-C3 can be positive	Extravascular adhesion to macrophages via FcγR and phagocytosis
	Drug binds loosely to erythrocyte → antibody forms to epitopes of drug and cell → activates complement cascade → antibody leaves cell	Polyspecific AHG positive, anti-IgG negative, anti-C3 positive	Intravascular complement-mediated lysis
Drug independent Autoantibody-like	Drug adheres to cell membrane → antibody forms primarily against epitopes on erythrocyte membrane → antibody reacts with erythrocyte	Polyspecific AHG positive, anti-IgG positive, anti-C3 positive or negative	Extravascular adhesion to macrophages via FcγR and phagocytosis
Nonimmune protein adsorption	Modification of cell membrane that results in nonimmunologically absorbed IgG, IgA, IgM, C3	Polyspecific AHG positive, monospecific can be positive or negative	Hemolysis can result

CASE STUDY *(continued from page 355)*

Two days later, Nancy's hemoglobin dropped to 50 g/L. The physician ordered several more tests. She had a positive IAT, and the antibody reacted with all cells including her own. Other test results indicated that this patient had systemic lupus erythematosus.

4. What type of antibody appears to be present in Nancy? Explain.
5. What is the relationship of Nancy's primary disease, systemic lupus erythematosus, and her anemia?

ALLOIMMUNE HEMOLYTIC ANEMIA

Hemolytic anemia induced by an individual's immunization with erythrocyte antigens on the infused cells of another individual is known as *alloimmune hemolytic anemia*. The patient's erythrocytes lack the antigen(s) present on infused cells. These transfused antigens are recognized as foreign and induce the recipient to form antibodies that, in turn, react with the transfused cells. This type of immunologic destruction of erythrocytes is characteristic of transfusion reactions and hemolytic disease of the fetus and newborn (HDFN). Factors such as the immunogenicity of the antigen, number of transfusions, and function of the recipient's immune system can influence the development of alloantibodies.[75]

In recent years, there has been evidence of the production not only of alloantibodies in patients undergoing solid organ and allogeneic bone marrow/stem cell transplants but also of autoantibodies.[76–78] Post-transplant immune-mediated hemolysis can be linked to major blood group incompatibility, passive transfer of antibody from the donor, development of autoimmunity, or passenger lymphocyte syndrome. *Passenger lymphocyte syndrome* is an immune hemolytic process that develops following solid organ, bone marrow, or stem cell transplant. The donor B lymphocytes transplanted with the organ or bone marrow produce antibodies against the recipient's blood group antigens. Although ABO incompatibility is most frequently implicated, other blood group systems can be involved. Development of autoantibodies after solid organ transplant is rare but is more common after bone marrow or stem cell transplants.[34,35] Some evidence also suggests that blood transfusions can in some patients lead to the development of autoantibodies.[77,78]

Hemolytic Transfusion Reactions

Transfusion of blood can cause a **hemolytic transfusion reaction** as the result of the interaction of foreign (nonself) antigens on transfused erythrocytes and alloantibodies in the patient's plasma. In contrast to AIHA, the antibodies produced in transfusion reactions cause immunologic destruction of donor cells but do not react with the erythrocytes of the person making the antibody. The two types of transfusion reactions involving alloantibodies to erythrocyte antigens are immediate (occurring within 24 hours) and delayed (occurring 2–14 days after transfusion) (Table 19-11 ★). An acute or immediate hemolytic transfusion reaction results when the infused erythrocytes react with antibodies that already exist in the recipient, usually ABO system antibodies, although other system antibodies have been implicated. This

★ **TABLE 19-11** Comparison of Acute and Delayed Hemolytic Transfusion Reactions

	Acute	Delayed
Timing	Immediate (within 24 hours)	2–14 days
Underlying cause	Usually ABO antibodies	Other antibodies: often Kidd system (anamnestic response)
Hemolysis	Intravascular	Extravascular; rare, slower intravascular if antibody capable of fixing complement
Symptoms	Fever, chills, back pain, hypotension, pain at site of infusion	Uncommon (fever, hemoglobinuria)
Laboratory findings	Hemoglobinemia	Positive DAT
	May see hemoglobinuria	
	Positive DAT (possible)	Antibody in eluate

type of reaction usually is caused by clerical or other human error that results in an ABO mismatch between donor and recipient. For example, the patient is given the wrong unit of blood or is misidentified by the phlebotomist, nurse, or laboratory personnel. Most errors occur in patient care areas, not in the transfusion service area. Patients given the wrong blood exhibit classic clinical signs including increased pulse rate, hypotension, chills and fever, pain, or difficulty breathing. When an acute transfusion reaction is suspected, the transfusion must be stopped immediately because of the potential complications of the reaction (e.g., shock, renal failure, disseminated intravascular coagulation [DIC]). Laboratory investigation of the reaction must be performed.

The delayed hemolytic transfusion reaction is usually the result of an anamnestic response in which the donor erythrocytes contain an antigen to which the patient has been previously sensitized. In these cases, the antibody was not detectable prior to transfusion, but the infused erythrocytes restimulate antibody production, destroying the donor cells containing the antigen. In a delayed transfusion reaction, some patients show no clinical signs, and the reaction is detected when laboratory tests demonstrate that the expected increase in hemoglobin has not occurred. Other patients can present with dark urine or vague symptoms such as fever. Patients at highest risk for delayed reactions are those who have received multiple transfusions over their lifetime.[75]

Pathophysiology

An acute hemolytic transfusion reaction is characterized by intravascular hemolysis with hemoglobinuria as a result of complete activation of the complement cascade. IgM antibodies usually mediate this type of hemolysis of donor cells, although IgG antibodies are rarely involved. Acute hemolytic transfusion reaction is typical of an ABO incompatibility and begins very shortly after the infusion of the donor unit has begun. As cells are lysed, the release of erythrocyte membrane phospholipids can activate the intrinsic and extrinsic coagulation cascade (Chapter 32). The resulting consumptive coagulopathy (disseminated intravascular coagulation) can damage the kidney by deposition of fibrin in the microvasculature (Chapters 32, 34). Presence of increased tumor necrosis factor-α (TNF-α) and other interleukins can mediate some of the clinical symptoms.[76] Mortality rates from ABO acute hemolytic transfusion reactions range from 10–50%.

Extravascular hemolysis is typical of a delayed hemolytic transfusion reaction occurring when erythrocytes are coated with IgG antibodies and removed via macrophage Fc receptors in the spleen. The speed of the removal depends on the amount of antibody on the cell. Complement is not usually involved but when present can enhance phagocytosis.

Delayed transfusion reactions occur 2–14 days after a transfusion. Although the antibody cannot be detected in pretransfusion testing because its concentration is lower than the sensitivity level of the test, antigens on infused donor cells induce a secondary (anamnestic) antigenic response. The antibody produced is usually IgG, and hemolysis is extravascular. The first indication of a delayed reaction is a sharp drop in the hemoglobin concentration several days after the transfusion with no signs of bleeding. Intravascular hemolysis can also occur but is less pronounced than in acute reactions. Laboratory investigation reveals a positive DAT because of antibody-coated donor cells in the patient's circulation. Antibodies characteristically associated with a delayed transfusion reaction are in the Kidd system (anti-Jk^a, anti-Jk^b), although antibodies to other antigens have also caused delayed reactions.

Clinical Findings

Symptoms of an immediate transfusion reaction begin within minutes to hours after beginning the transfusion. The reaction between antigen and antibody can trigger cytokine release, and activation of the complement and the coagulation cascades. A variety of nonspecific symptoms, including fever, chills, low back pain, sensations of chest compression or burning at the site of infusion, hypotension, nausea, and vomiting, can occur. Unless the transfusion is immediately stopped, shock can occur. Anuria due to tubular necrosis secondary to inadequate renal blood flow and bleeding due to DIC are common complications. The severity of the reaction and extent of organ damage are directly proportional to the amount of blood infused.

Most delayed transfusion reactions cause few signs or symptoms. The most common signs are malaise and unexplained fever several days after the transfusion. Some patients notice the presence of hemoglobin in the urine.

☑ CHECKPOINT 19-12

Compare the underlying mechanisms, pathophysiology, and clinical symptoms of an acute hemolytic transfusion reaction with a delayed one.

Laboratory Findings

The laboratory findings vary, depending on whether the transfusion reaction is acute or delayed. Intravascular hemolysis usually accompanies the acute reaction and typically can be associated with hemoglobinemia, hemoglobinuria, methemoglobinemia, and decreased haptoglobin and hemopexin. Extravascular hemolysis usually accompanies the delayed reaction. The DAT might or might not be positive in acute hemolytic transfusion reactions, depending on the extent of erythrocyte destruction. The DAT is usually positive in a delayed hemolytic transfusion reaction but might not be detected until days after transfusion. A patient who is suspected of experiencing either an acute or delayed transfusion reaction should have a transfusion reaction workup. See the supplemental Web material for Chapter 19 for further discussion on a typical transfusion reaction workup.

☑ CHECKPOINT 19-13

Compare the characteristic laboratory findings in acute hemolytic transfusion reactions and in delayed transfusion reactions.

Therapy

The most important immediate action to take when an acute transfusion reaction occurs is to terminate the transfusion. A major effort should be made to maintain urine flow to prevent renal damage. Shock and bleeding require immediate attention. In a delayed reaction, treatment is generally not required. Future units of blood given to the patient, however, must lack the antigen to which the patient has made an antibody.

CASE STUDY (continued from page 363)

The clinician wants to start Nancy's therapy and give her a transfusion.

6. How would knowing that Nancy had not been transfused in the last several months help you make a decision on the underlying cause of the antibody?
7. What would you tell the clinician about giving a transfusion?
8. What kind of therapy could be used?

Hemolytic Disease of the Fetus and Newborn (HDFN)

Hemolytic disease of the fetus and newborn (HDFN) is an alloimmune disease associated with increased erythrocyte destruction during fetal and neonatal life and is caused by fetomaternal blood group incompatibility. The three categories of HDFN are: ABO caused by anti-A, anti-B, and/or anti-A, B; Rh(D) caused by anti-D; and "other" caused by alloantibodies to other Rh system antigens (C, c, E, e) or antibodies to other blood group system antigens (e.g., Kell, Kidd, Duffy). More than 95% of HDFN cases are due to either anti-D or ABO system antibodies. Although HDFN caused by ABO antibodies is more common than HDFN caused by anti-D, Rh(D) incompatibility causes more severe disease (Table 19-12 ★). The antibodies most commonly associated with the remaining HDFN cases include anti-K, anti-c, anti-C, and anti-E, although any IgG antibody can be implicated. HDFN caused by anti-D and anti-K are the most severe.[2,79]

Pathophysiology

The pathophysiology of HDFN involves initial sensitization and antibody production, in utero effects, and postnatal effects. Four conditions must be met for HDFN to occur:

- The mother must be exposed (sensitized) to an erythrocyte antigen that she lacks.
- The fetus must possess the antigen to which the mother has been sensitized.
- The mother must produce antibodies to the foreign antigens.
- The mother's antibody must be able to cross the placenta and enter the fetal circulation

Sensitization

The mother may have been exposed to foreign (nonself) erythrocyte antigens by previous pregnancy or transfusion. Normally, the placenta does not allow free passage of erythrocytes from fetal to maternal circulation, but small numbers of erythrocytes can enter the maternal circulation during gestation. Additionally, small amounts of fetal blood also can enter the mother's circulation during delivery. The risk of sensitization increases as the volume of the fetal bleed increases. If the fetal-maternal bleed is sufficient to stimulate the production of maternal antibodies, subsequent pregnancies could be at risk for HDFN.

Although three classes of immunoglobulins can be produced during the mother's immunization—IgG, IgM, IgA—only IgG has the ability to cross the placenta and cause HDFN. The IgG antibody is actively transported across the placenta and causes destruction of fetal erythrocytes. The fetus/newborn has anemia and bilirubinemia of varying degrees of severity based on the strength of the immune response and degree of hemolysis.

In ABO-HDFN, the mother already has the naturally occurring ABO system antibodies, generally a mixture of IgM and IgG anti-A, anti-B, and/or anti-A,B. The pre-existing IgG antibodies can cross the placenta to destroy fetal cells, potentially affecting all pregnancies including the first. Approximately 15–20% of the group A or group B babies born to group O mothers develop serologic evidence of ABO-HDFN (positive DAT).[80] In HDFN caused by all other antibodies including anti-D, the mother generally lacks preformed antibodies capable of recognizing fetal antigens. The firstborn is not usually affected because the first pregnancy serves as the sensitizing event. In each subsequent pregnancy with an antigen-positive fetus, the risk for severity of HDFN increases as the antibody response increases.

Prenatal Period

If destruction of fetal erythrocytes is severe enough in utero, the fetus becomes severely anemic and can develop complications as a result. Extramedullary hematopoiesis occurs in the liver and spleen, causing

★ **TABLE 19-12** Comparison of Hemolytic Disease of the Fetus and Newborn Caused by ABO and Rh(D)

Feature	Rh	ABO	Other
Antibody	Immune IgG	Nonimmune IgG	Immune IgG
Blood group	Mother Rh negative Baby Rh positive	Mother, group O; newborn, group A or B	Mother lacks antigen that is on fetal cell
Obstetric history	Only pregnancies after the first are usually affected	First pregnancy and subsequent pregnancies can be affected	Pregnancy can be first if mother previously sensitized by transfusion. If pregnancy is sensitizing event, usually second and subsequent pregnancies affected
Clinical findings	Moderate to severe anemia and bilirubinemia	Mild anemia if present; mild to moderate bilirubinemia with a peak 24–48 hours after birth	Mild to severe anemia and bilirubinemia
Laboratory findings	DAT positive No spherocytes	DAT weakly positive or negative Spherocytes present	DAT positive
Therapy	Exchange transfusion if severe	Phototherapy	Phototherapy and/or exchange transfusion, if severe

their enlargement. Because of hemolysis, the unconjugated (indirect) bilirubin concentration increases. In the fetus, this bilirubin crosses the placenta and is conjugated and excreted by the mother. With procedures such as amniocentesis, the amount of bilirubin in amniotic fluid can be measured to help determine the relative severity of hemolysis. Cordocentesis in which a sample of the fetal blood is taken via a needle inserted into the umbilical vein allows testing of the fetal blood for blood type, DAT, hemoglobin, and bilirubin. Noninvasive procedures such as Doppler ultrasound can help predict anemia based on blood flow in the fetus.[81–83] In recent years, methods to obtain fetal cells or cell-free DNA from the maternal circulation have been introduced. These methods help in predicting Rh-HDFN and eliminating the need for invasive procedures such as amniocentesis. Prenatal fetal genotyping diagnostic procedures have been developed. Although they were initially designed to detect the D antigen, they have been expanded to detect K, Fy, and JK genotypes as well.[82–84]

The most serious complication of HDFN is cardiac failure and hydrops fetalis, which occurs when the fetus is unable to produce sufficient erythrocytes. Hydrops fetalis is characterized by edema and accumulation of fluid in the peritoneal, pericardial, or pleural cavities.

Erythroblastosis fetalis, a term also used to describe characteristics of HDFN, reflects the presence of large numbers of nucleated erythrocytes found in the newborn's peripheral blood in very severe cases.

Postnatal Period

Erythrocyte destruction persists after birth because of maternal antibodies in the newborn's circulation. After birth, the newborn must conjugate and excrete the bilirubin on its own. In the neonate, albumin levels for bilirubin transport are limited, and liver glucuronyl transferase for bilirubin conjugation is low; therefore, considerable amounts of toxic unconjugated bilirubin can accumulate in the newborn after delivery. In the unconjugated state, bilirubin is toxic because it is lipid soluble and can easily cross cell membranes. This form of bilirubin has a high affinity for basal ganglia of the central nervous system. Thus, the excess unconjugated bilirubin can lead to **kernicterus**, an irreversible form of brain damage. The conjugated form of bilirubin cannot cause this problem because it is water soluble and lipid insoluble and cannot cross cell membranes.

Clinical Findings

Anemia resulting from increased cell destruction is the greatest risk to both the fetus in utero and to the newborn with HDFN in the first 24 hours of life. Bilirubinemia is the greatest risk thereafter. In Rh(D) incompatibility, the cord blood hemoglobin can be low normal at birth (normal hemoglobin at birth is 14–20 g/dL), and the newborn may not appear jaundiced. However, significant hemolysis occurring in the first 24 hours of life outside the womb results in anemia with pallor and jaundice. In severe cases, hepatosplenomegaly can be present. Severe anemia can be accompanied by heart failure and edema. As the level of unconjugated bilirubin rises, kernicterus can occur and can be fatal in severe cases. The risk of hyperbilirubinemia in premature infants is even greater because of the inability of the premature liver to excrete the excess bilirubin.

ABO incompatibility is not as severe as Rh incompatibility. The clinical course is usually benign, and hemolysis is minimal. Within 24–48 hours after birth, the infant appears jaundiced, but kernicterus is extremely rare. Anemia is mild and pallor is uncommon. Hepatosplenomegaly, if present, is mild. However, cases of severe ABO-HDFN requiring exchange transfusion as well as phototherapy, especially in blacks and children of mixed race parents, have been reported.[80]

☑ CHECKPOINT 19-14

Compare the pathophysiology and clinical findings of ABO-HDFN and Rh-HDFN.

Laboratory Findings

Laboratory tests are essential to identify the etiology of HDFN, determine prognosis, and select appropriate treatment. Classic prenatal testing on a pregnant woman includes ABO and Rh typing of the erythrocytes as well as an antibody screen (IAT) on her serum. If the IAT is positive, the antibody is identified so that an assessment of HDFN risk can be performed. The postnatal HDFN workup will involve laboratory tests on both the mother and the infant.

Rh Incompatibility

The DAT in Rh incompatibility is usually positive, reflecting antibody coating of the newborn's erythrocytes.[85] About 50% of affected infants have a cord blood hemoglobin concentration of <14 g/dL. Because the capillary blood hemoglobin can be up to 4 g/dL higher due to placental transfer of blood at birth, the cord blood hemoglobin concentration is most useful as an indicator of anemia at birth and a baseline to follow destruction of erythrocytes after birth. A direct relationship exists between the initial cord blood hemoglobin level and the severity of the disease. Lower cord hemoglobin levels at birth are associated with a more severe clinical course. After birth, hemoglobin levels can fall at the rate of 3 g/dL/day. Lowest hemoglobin values are present at 3–4 days. The erythrocytes are macrocytic and normochromic. Reticulocytes are markedly increased, sometimes reaching 60%. Nucleated red cells are markedly increased in the peripheral blood ($10–100 \times 10^9$/L) reflecting the rapid production of cells in response to erythrocyte destruction. Normal infants also have nucleated red cells in the peripheral blood, but their values are much lower ($0.2–2.0 \times 10^9$/L).

A blood smear shows marked polychromasia, mild or absent poikilocytosis, and few, if any, spherocytes. In some cases, the leukocyte count is increased to 30×10^9/L or more due to a rise in neutrophils reflecting the marrow response to stress. (The normal leukocyte count at birth is $15–20 \times 10^9$/L.) However, recent studies have shown that a neutropenia is often present, regardless of the type of HDFN.[86] A significant neutrophilic shift to the left often occurs. The platelet count is usually normal, but thrombocytopenia can develop in severe cases.

Cord blood bilirubin is elevated in Rh-HDFN but is usually <5.5 mg/dL. However, the elevated bilirubin does not accurately reflect the severity of hemolysis because bilirubin produced before birth readily crosses the placenta and is metabolized by the mother. The newborns serum bilirubin peaks on the third or fourth day and can reach 40–50 mg/dL if not treated. Most bilirubin is in the toxic

unconjugated form. Full-term infants with bilirubin concentrations >10 mg/dL are at increased risk for kernicterus, and premature infants can develop it with levels as low as 8–10 mg/dL.

ABO Incompatibility

In the case of ABO incompatibility, weakly positive DAT is found in the cord blood, but it often becomes negative within 12 hours. The weak reaction is due to the small number of anti-A or anti-B antibodies attached to the erythrocyte. Bilirubin is not usually significantly elevated, but the bilirubin level 6 hours after birth can be used to predict development of hyperbilirubinemia in severe cases.[87] The peripheral blood smear in severe cases can show increased numbers of nucleated erythrocytes and the presence of schistocytes, spherocytes, and polychromasia.

☑ CHECKPOINT 19-15

Compare the laboratory findings including the peripheral blood smear and the DAT for newborns with ABO-HDFN and those with Rh-HDFN.

Therapy

The major efforts of therapy are to prevent hyperbilirubinemia and anemia. If the destruction of erythrocytes and degree of anemia appear to affect the viability of the fetus, an intrauterine transfusion can be given. Methods such as amniocentesis and cordocentesis and now the use of molecular methods to identify fetal DNA for detecting in utero hemolysis have allowed decisions to be made on whether an intrauterine transfusion should be given.[84] Transfusions often must be given on a routine basis until the fetus reaches a gestational age that allows successful delivery.

In mild cases postnatally, the newborn is treated with phototherapy, which slowly lowers the toxic bilirubin level. Although toxic levels of bilirubin are 19–20 mg/dL, exchange transfusion is usually performed before that level is reached. Exchange transfusions can also be indicated if the bilirubin is rising >1 mg/dL/hour or if there is significant anemia. The transfusion has several beneficial effects:

- Removes plasma containing maternal antibodies and dilutes the concentration of remaining antibodies
- Removes some of the antibody-coated erythrocytes
- Lowers the level of bilirubin
- Treats the anemia

Rh Immune Globulin (RhIG)

The passive injection of Rh immunoglobulin (RhIG) that contains anti-D prevents maternal immunization. About 7–8% of Rh-negative women develop antibodies to Rh-positive cells after the birth of an Rh-positive ABO-compatible infant. The routine use of prophylactic RhIG in Rh-negative women during gestation (at 28 weeks) and following the birth of an Rh-positive child has decreased the incidence of HDFN considerably, although other changes in prenatal care and number of pregnancies also may have affected the incidence. The majority of women (92%) who develop anti-D during pregnancy do so at 28 weeks or later. Therefore, antepartum administration of RhIG is given between weeks 28 and 30 of gestation to any Rh-negative woman who has not developed anti-D. The RhIG acts as an immunosuppressant, depressing the production of immune IgG. However, this prenatal anti-D binds to fetal cells and can result in a positive DAT and must be evaluated for clinical and serological significance.[88]

Postnatally, a dose of RhIG given within 72 hours of birth protects against the consequences of a fetal-maternal bleed. Rh-positive erythrocytes of a fetus that enter the mother's circulation at birth can stimulate the mother's immune system to make antibodies. As in the antepartum administration of RhIG, postnatal RhIG can bind fetal cells and mediate their removal in the spleen. The dose of RhIG should be determined based on the number of fetal cells in the maternal circulation. Several tests can be used, including the Rosette and the Kleihauer-Betke tests, and, more recently, immunofluorescent flow cytometry using monoclonal antibody to fetal hemoglobin to determine the number of fetal cells present.

Summary

Immune hemolytic anemia (IHA) is mediated by antibodies and/or complement and can be classified as autoimmune, alloimmune, or drug induced, depending on the underlying process. All of these have a positive DAT due to immunoglobulin and/or complement on the cell. The autoimmune hemolytic anemias (AIHA) occur when the offending antibody reacts with an antigen on the patient's erythrocytes (self-antigen). These antibodies also have the ability to react with erythrocytes of most other persons. AIHA are either idiopathic or secondary to an underlying disease and are further classified as warm or cold, depending on the thermal reactivity of the causative antibody. WAIHA is most often caused by IgG antibodies that react at body temperature (37°C) and cause extravascular hemolysis. Cold hemagglutinins are IgM antibodies that generally react at <20°C but are efficient complement activators and can cause intravascular hemolysis. In both cases, the DAT is positive, and the IAT procedure can detect the antibody. An IgG biphasic antibody that binds complement in the cool peripheral circulation mediates PCH, another type of cold autoimmune hemolytic condition. As the coated cells reach warmer portions of the circulation, the complement cascade is activated and intravascular hemolysis occurs.

In drug-induced immune hemolytic anemia, the drug binds to the erythrocyte membrane and induces antibody formation, which occurs by several mechanisms, depending on the causative drug. The antibodies can be drug dependent, and the drug must be present for the antibody to react. In autoantibody formation (drug independent), the drug appears to cause a change in the erythrocyte membrane that causes the body to recognize it as foreign and produce an antibody against the cell.

Alloimmune hemolytic anemia has two presentations: transfusion reaction and hemolytic disease of the newborn. In both conditions, infusion of erythrocytes containing foreign (nonself) antigens stimulates the antibody. This sensitization causes the production of alloantibodies to the foreign antigens, resulting in the cell's destruction. The antibody can be detected in the serum and cause a positive DAT.

Transfusion reactions can be acute (immediate) or delayed (2–14 days after transfusion). Acute reactions most often involve an ABO mismatch between donor and recipient and result in intravascular hemolysis as a result of complement activation. Delayed transfusion reactions are due to non-ABO antibodies. These reactions result in extravascular lysis and are characterized by a positive DAT. The causative antibody must be identified so that future transfused erythrocytes lack the antigen corresponding to the patient's antibody.

ABO, Rh(D) or other blood group system antibodies that react with fetal erythrocytes can cause hemolytic disease of the fetus and newborn (HDFN). ABO-HDFN is generally milder than Rh-HDFN and can occur in any pregnancy because prior antigenic sensitization is not required. Rh and other types of HDFN require prior sensitization. Maternal antibodies that coat fetal cells can cause anemia and/or elevated bilirubin in the newborn. Maternal and newborn ABO and Rh group, maternal antibody, and newborn DAT are used to evaluate HDFN.

Review Questions

Level I

1. The characteristic erythrocyte seen in a peripheral blood smear in WAIHA is a(n): (Objective 3)
   A. macrocyte
   B. spherocyte
   C. dacrocyte
   D. elliptocyte
2. Which of the following might be observed on a peripheral blood smear in cases of cold autoimmune hemolytic anemia? (Objective 3)
   A. helmet cells
   B. macrocytes
   C. agglutination
   D. spherocytes
3. Which of the following parameters on an automated hematology instrument could be seen in cases of cold agglutinin disease (CAD)? (Objective 3)
   A. falsely elevated MCV
   B. falsely elevated RBC count
   C. falsely decreased MCHC
   D. falsely decreased hemoglobin
4. One purpose of the DAT is to: (Objective 2)
   A. detect erythrocytes coated with immunoglobulin in vivo
   B. detect antibodies in the serum
   C. neutralize serum complement
   D. prevent agglutination by IgM antibodies
5. Intravascular hemolysis is characteristic of which of the following alloantibody situations? (Objective 5)
   A. delayed hemolytic transfusion reaction
   B. ABO-HDFN
   C. Rh-HDFN
   D. acute hemolytic transfusion reaction
6. A patient with WAIHA would most likely have serum that reacts in which of these patterns? (Objective 5)

	Serum + Own Erythrocytes	Serum + Erythrocytes of Others
A.	negative	negative
B.	negative	positive
C.	positive	positive
D.	positive	negative

7. Which of the following is *not* considered to be a condition caused by autoantibodies? (Objective 5)
   A. PCH
   B. CAS
   C. delayed transfusion reaction
   D. drug-induced hemolytic anemia
8. The Donath-Landsteiner antibody is found in which type of hemolytic anemia? (Objective 1)
   A. warm autoimmune hemolytic anemia
   B. paroxysmal cold hemoglobinuria
   C. cold autoimmune hemolytic anemia
   D. drug-induced hemolytic anemia
9. Which of the following is *not* part of the typical peripheral blood picture in Rh-HDFN? (Objective 4)
   A. macrocytes
   B. polychromasia
   C. spherocytes
   D. increased nucleated erythrocytes
10. The antibody found in PCH is: (Objective 1)
    A. IgM
    B. IgG
    C. directed against the Rh antigens
    D. directed against the ABO antigens

Level II

1. The drug(s) most often associated with drug-induced hemolytic anemia is/are: (Objective 3)

   A. aldomet
   B. second and third generation cephalosporins
   C. penicillin
   D. rituximab

2. Because a newborn shows evidence of jaundice, a workup for HDFN is started. The newborn has a weakly positive DAT with anti-IgG. The mother is group O, Rh negative., and the newborn is group A, Rh negative. The blood smear shows evidence of spherocytes. What is the most likely cause? (Objective 6)

   A. Rh-HDFN
   B. ABO-HDFN
   C. combined ABO- and Rh-HDFN
   D. cold agglutinins

3. Which autoimmune syndrome is characterized by the presence of a biphasic complement-fixing IgG antibody? (Objective 4)

   A. PCH
   B. WAIHA
   C. cold agglutinin disease
   D. immune complex drug induced

4. Intravenous administration of what drug is characteristically associated with covalent attachment of the drug to the erythrocyte membrane and drug-dependent antibody formation? (Objective 3)

   A. penicillin
   B. aldomet
   C. quinidine
   D. third-generation cephalosporins

5. Based on maternal and fetal testing, a newborn is suspected of having Rh-HDFN. Which of the following peripheral blood smear morphologies might be seen? (Objective 6)

   A. microcytic, hypochromic erythrocytes, decreased reticulocytes
   B. microcytic, normochromic erythrocytes, increased nucleated red blood cells
   C. macrocytic, hypochromic erythrocytes, decreased reticulocytes
   D. macrocytic, normochromic erythrocytes, increased nucleated red blood cells

6. What is the *most likely* mechanism of hemolysis in WAIHA? (Objective 1)

   A. increased sensitivity to complement
   B. fixing of complement by IgM antibody
   C. biphasic reactions by IgG antibodies
   D. phagocytosis of IgG coated erythrocytes

7. A patient who received a transfusion 6 days ago is suspected of having a delayed transfusion reaction. Which of the following would *not* be a characteristic finding? (Objective 7)

   A. positive DAT
   B. positive IAT
   C. hemoglobinuria
   D. decreased hemoglobin

8. How would you interpret the following results of a Donath-Landsteiner test? (Objective 4)

Patient incubated at 4°C and then 37°C	Hemolysis
Control incubated only at 37°C	No hemolysis

   A. positive
   B. negative
   C. invalid because of control reaction
   D. equivocal—repeat in 2 weeks

9. A 75-year-old man presents to the physician with complaints of weakness and fatigue. His leukocyte count is elevated, and his hemoglobin is 60 g/L. The peripheral blood smear shows that a majority of the cells are small, mature lymphocytes. The diagnosis is CLL. Spherocytes are present. The physician suspects hemolytic anemia, and laboratory tests suggest a hemolytic process with an increase in reticulocytes and bilirubin. Based on this information, what is the most likely problem? (Objective 8)

   A. secondary CAD
   B. secondary PCH
   C. secondary WAIHA
   D. secondary cold agglutinins

10. The following DAT results were seen in a 35-year-old female who was being investigated for a drug-induced hemolytic anemia. She had a hemoglobin of 70 g/L, and her serum reacted with all cells against which it was tested. What drug is the most likely cause? (Objective 3)

DAT (polyspecific)	Positive
DAT anti-IgG	Positive
DAT anti-C3	Negative

   A. penicillin
   B. quinidine
   C. cephalosporin
   D. fludarabine

Companion Resources

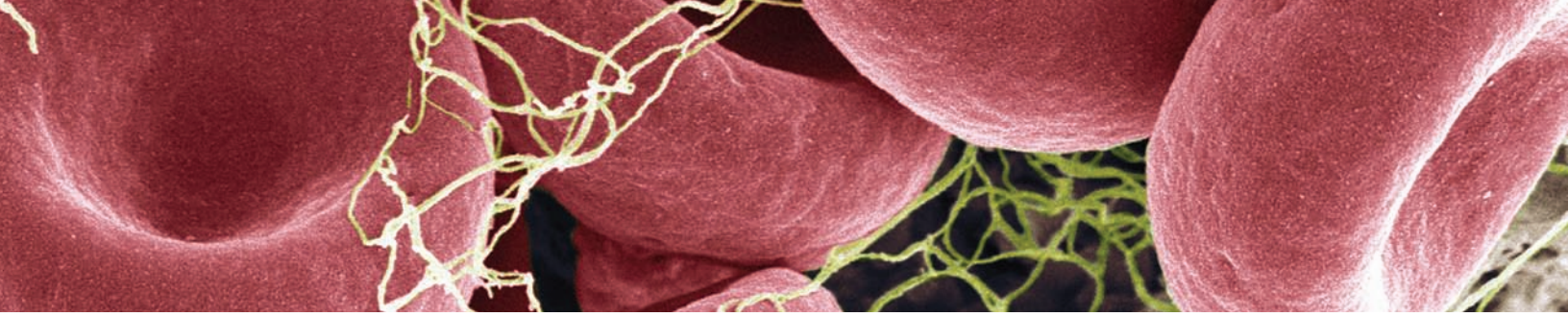
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Petz LD, Garratty G. *Immune Hemolytic Anemias*, 2nd ed. Philadelphia: Churchill Livingstone; 2004.
2. Roback JD, Grossman BJ, Harris T et al., eds. *Technical Manual*, 17th ed. Arlington, VA: AABB; 2011.
3. Garratty G. Immune hemolytic anemia: a primer. *Semin Hematol*. 2005;42:119–21.
4. Sokol RJ, Booker DJ, Stamps R et al. IgA red cell autoantibodies and autoimmune hemolysis. *Transfusion*. 1997;37:175–81.
5. Arndt PA, Leger RM, Garratty G. Serologic findings in autoimmune hemolytic anemia associated with immunoglobulin M warm autoantibodies. *Transfusion*. 2009;49:235–42.
6. Bleakly NT, Fontaine MJ, Pate LL et al. Disseminated intravascular coagulation due to IgM-mediated autoimmune hemolytic anemia. *Pediatr Blood Cancer*. 2011;57:329–31.
7. Arndt PA, Garratty, G. The changing spectrum of drug-induced immune hemolytic anemia. *Semin Hematol*. 2005;42:137–44.
8. Wikman A, Axdorph U, Gryfelt G et al. Characterization of red cell autoantibodies in consecutive DAT-positive patients with relation to in vivo haemolysis. *Ann Hematol*. 2005;84:150–58.
9. Azerda daSilveira S, Kikuchi S, Fossati-Juimak L et al. Complement activation selectively potentiates the pathogenicity of the IgG2 and IgG3 isotypes of a high affinity anti-erythrocyte autoantibody. *J Exp Med*. 2002;195:665–72.
10. Ruiz-Arguelles A, Llorente L. The role of complement regulatory proteins (CD55 and CD59) in the pathogenesis of autoimmune hemocytopenia. *Autoimmun Rev*. 2007;6:155–61.
11. Garratty G. The James Blundell Award Lecture 2007: Do we really understand immune red cell destruction? *Transfus Med*. 2008;18:321–34.
12. Nielsen CH, Fischer EM, Leslie RGQ. The role of complement in the acquired immune response. *Immunology*. 2000;100:4–12.
13. Sachs UHJ, Roder L, Santoso S et al. Does a negative direct antiglobulin test exclude warm autoimmune haemolytic anaemia? A prospective study of 504 cases. *Br J Haematol*. 2002;132:655–56.
14. Garratty G. Immune hemolytic anemia associated with negative routine serology. *Semin Hematol*. 2005;42:156–64.
15. Reardon JE, Marques MB. Laboratory evaluation and transfusion support of patients with autoimmune hemolytic anemia. *Am J Clin Pathol*. 2006;125S1:S71–77.
16. Sokol RJ, Booker DJ, Stamps R et al. Direct Coombs test—negative autoimmune hemolytic anemia and low-affinity IgG class antibodies. *Immunohematology*. 1997;13:115–18.
17. Chaudhary R, Das SS, Gupta R et al. Application of flow cytometry in detection of red-cell-bound IgG in Coombs-negative AIHA. *Hematology*. 2006;11:295–300.
18. Lai M, Rumi C, D'Onofrio G et al. Clinically significant autoimmune hemolytic anemia with a negative direct antiglobulin test by routine tube test and positive by column agglutination method. *Immunohematology*. 2002;18:109–13.
19. Bartolmas T, Salama A. A dual antiglobulin test for the detection of weak or nonagglutinating immunoglobulin M warm autoantibodies. *Transfusion*. 2010;50:1131–34.
20. Parman P. *The Immune System*, 3rd ed. Abingdon, UK: Garland Science Publishing; 2009.
21. Ghers BC, Friedberg RC. Autoimmune hemolytic anemia. *Am J Hematol*. 2002;69:258–71.
22. Vaglio S, Arista MC, Perrone MP et al. Autoimmune hemolytic anemia in childhood: serologic features in 100 cases. *Transfusion*. 2007;47:50–54.
23. Hodgson K, Ferrer G, Pereira A et al. Autoimmune cytopenia in chronic lymphocytic leukaemia: diagnosis and treatment. *Br J Haematol*. 2011;154:14–22.
24. Mauro FR, Foa R, Cerretti R et al. Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features. *Blood*. 2000;95:2786–92.
25. Kokori SIG, Ionnidis JPA, Voulgarelis M et al. Autoimmune hemolytic anemia in patients with systemic lupus erythematosus. *Am J Med*. 2000;108:198–209.
26. Plikat K, Rogler G, Scholmerich J. Coombs-positive autoimmune hemolytic anemia in Crohn's disease. *Eur J Gastroenterol Hepatol*. 2005;17:661–66.
27. Domiciano DS, Shinjo SK. Autoimmune hemolytic anemia in systemic lupus erythematosus: association with thrombocytopenia. *Clin Rheumatol*. 2010;29:1427–31.
28. Kao YS, Kirkley KC. A patient with mixed connective tissue disease and mixed-type autoimmune hemolytic anemia. *Transfusion*. 2005;45:1695–96.
29. Nobili V, Vento S, Comparcola D et al. Autoimmune hemolytic anemia and autoimmune hepatitis associated with Parvovirus B19 infection (letter). *Pediatr Infect Dis J*. 2004;23:184–85.
30. Sevilla J, delCarmen Escudero M, Jimenez R et al. Severe systemic autoimmune disease associated with Epstein-Barr virus infection. *J Pediatr Hematol Oncol*. 2004;26:831–33.
31. Mason HM, Arndt PA. A 13-year-old girl with cold agglutinin syndrome caused by anti-i. *J Pediatr Hematol Oncol*. 2008;30:543–45.
32. Downes KA, Domen RE, McCarron KF et al. Acute autoimmune hemolytic anemia following DPT vaccination: report of a fatal case and review of the literature. *Clin Pediatr*. 2001;40:355–58.
33. Seltsam A, Shukry-Schulz S, Salama A. Vaccination-associated immune hemolytic anemia in two children. *Transfusion*. 2000;40: 907–9.
34. Li M, Goldfinger D, Yuan S. Autoimmune hemolytic anemia in pediatric liver or combined liver and small bowel transplant patients: a case series and review of the literature. *Transfusion*. 2012;52:48–52.
35. Milot T, Arnon R, Roman E et al. Autoimmune hemolytic anemia and idiopathic thrombocytopenic purpura in pediatric solid organ transplant recipients, report of five cases and review of the literature. *Pediatr Transplant*. 2011;15:870–78.
36. Wheeler CA, Calhoun L, Blackall DP. Warm reactive autoantibodies: clinical and serologic correlations. *Am J Clin Pathol*. 2004;122:680–85.
37. Barros MMO, Blajchman MA, Bordin JO. Warm autoimmune hemolytic anemia: recent progress in understanding the immunobiology and the treatment. *Transfus Med Rev*. 2010;24:195–210.
38. Buetens OW, Ness PM. Red blood cell transfusion in autoimmune hemolytic anemia. *Curr Opin Hematol*. 2003;10:429–33.
39. Lechner K, Jager U. How I treat autoimmune hemolytic anemias in adults. *Blood*. 2010;116:1831–38.
40. Shirey RS, Boyd JS, Parwini AV et al. Prophylactic antigen-matched donor blood for patients with warm autoantibodies: an algorithm for transfusion management. *Transfusion*. 2002;42:1435–41.
41. Crowther M, Chan YLT, Garbett IK et al. Evidence-based focused review of the treatment of idiopathic warm immune hemolytic anemia in adults. *Blood*. 2011;118:4036–40.
42. Zeelander S. Autoimmune haemolytic anaemia—a practical guide to cope with a diagnostic challenge. *Neth J Med*. 2008;69:177–84
43. D'Arena G, Laurenti K, Capalbo S et al. Rituximab therapy for chronic lympyhocytic leukemia-associated autoimmune hemolytic anemia. *Am J Hematol*. 2006;81:598–602.
44. Gertz MA. Cold hemolytic syndrome. *Hematology*. 2006;19–23.

45. Petz LD. Cold antibody autoimmune hemolytic anemias. *Blood Rev*. 2008;22:1–18.
46. McNicholl FP. Clinical syndromes associated with cold agglutinins. *Transfusion Sci*. 2000;22:125–33.
47. Ulvestad E, Berentsen S, Bo K et al. Clinical immunology of chronic cold agglutinin disease. *Eur J Haematol*. 1999;63:259–66.
48. Konig AI, Schabel A, Sugg U et al. Autoimmune hemolytic anemia caused by IgGλ-monotypic cold agglutinins of anti-Pr specificity after rubella infection. *Transfusion*. 2001;41:488–92.
49. Tereda K, Tanaka H, Mori R et al. Hemolytic anemia associated with cold agglutinin during chickenpox and a review of the literature. *J Pediatr Hematol Oncol*. 1998;20:149–51.
50. Koduri PR, Singa P, Nikolinakos P. Autoimmune hemolytic anemia in patients infected with human immunodeficiency virus-1. *Am J Hematol*. 2002;70:174–76.
51. Jubinshky PT, Rashid N. Successful treatment of a patient with mixed warm and cold antibody mediated Evans syndrome and glycose intolerance. *Pediatr Blood Cancer*. 2005;45:347–50.
52. Berentsen S, Ulvestad E, Gjertsen BT et al. Rituximab for primary chronic cold agglutinin disease: a prospective study of 37 courses of therapy in 27 patients. *Blood*. 2004;103:2925–28.
53. Berentsen S. How I manage cold agglutinin disease. *Br J Haematol*. 2011;153:309–17.
54. Eder AF. Review: acute Donath-Landsteiner hemolytic anemia. *Immunohematology*. 2005;21:56–62.
55. Sivakumaran M, Murphy PT, Booker DJ et al. Paroxysmal cold haemoglobinuria caused by non-Hodgkin's lymphoma. *Br J Haematol*. 1999;105:278–79.
56. Ziman A, Hsi R, Goldfinger D. Donath-Landsteiner antibody-associated hemolytic anemia after *Haemophilus influenzae* infection in a child. *Transfusion*. 2004;44:1127–28.
57. Chambers LA, Rauck AM. Acute transient hemolytic anemia with a positive Donath-Landsteiner test following Parvovirus B19 infection. *J Pediatr Hematol Oncol*. 1996;18:178–81.
58. Roy-Burman A, Glader BE. Resolution of severe Donath-Landsteiner autoimmune hemolytic anemia temporally associated with institution of plasmapheresis. *Crit Care Med*. 2002;30:931–34.
59. Garratty G. Erythrophagocytosis on the peripheral blood smear and paroxysmal cold hemoglobinuria (letter). *Transfusion*. 2001;41:1073.
60. Koppel A, Lim S, Osby M et al. Rituximab as successful therapy in a patient with refractory paroxysmal cold hemoglobinuria. *Transfusion*. 2007;47:1902–4.
61. Win N, Tiwari D, Keevil VL et al. Mixed-type autoimmune haemolytic anaemia: unusual cases and a case associated with splenic T-cell angioimmunoblastic non-Hodgkin's lymphoma. *Hematology*. 2007;12:159–62.
62. Mayer B, Yurak S, Kiesewetter H et al. Mixed type autoimmune hemolytic anemia: differential diagnosis and a critical review of reported cases. *Transfusion*. 2008;48:2229–34.
63. Morselli M, Luppi M, Potenza L et al. Mixed warm and cold autoimmune hemolytic anemia. Complete recovery after 2 courses of rituximab treatment. *Blood*. 2002;99:3478–79.
64. Garratty G. Drug induced immune hemolytic anemia. *Hematology*. 2009;73–79.
65. Garratty G. Immune hemolytic anemia associated with drug therapy. *Blood Rev*. 2010;24:143–50.
66. Naylor CS, Steele L, Hsi R et al. Cefotetan-induced hemolysis associated with antibiotic prophylaxis for cesarean delivery. *Am J Obstet Gynecol*. 2000;182:1427–28.
67. Seltsam A, Salama A. Ceftriaxone-induced immune haemolysis: two case reports and a concise review of the literature. *Intensive Care Med*. 2000;26:1390–94.
68. Arndt PA, Leger RM, Garratty G. Serology of antibodies to second- and third-generation cephalosporins associated with immune hemolytic anemia and/or positive direct antiglobulin tests. *Transfusion*. 1999;39:1239–46.
69. Arndt PA, Garratty G, Hill J et al. Two cases of immune haemolytic anaemia, associated with anti-piperacillin, detected by the "immune complex" method. *Vox Sang*. 2002;83:273–78.
70. Arndt PA, Garratty G. The changing spectrum of drug-induced immune hemolytic anemia. *Semin Hematol*. 2005;42:137–44.
71. Muellar-Eckhart C, Salama A. Drug-induced immunocytopenias: a unifying pathogenic concept with special emphasis on the role of drug metabolites. *Trans Med Rev*. 1990;4:69–77.
72. Habibi B. Drug-induced red blood cell autoantibodies co-developed with drug specific antibodies causing haemolytic anemias. *Br J Haematol*. 1985;61:139–43.
73. Pierce A, Nestor T. Pathology consultation on drug-induced hemolytic anemia. *Am J Clin Pathol*. 2011;136:7–12.
74. Garratty G, Arndt P, Prince HE et al. The effect of methyldopa and procainamide on suppressor cell activity in relation to red cell autoantibody production. *Br J Haematol*. 1993;84:310–15.
75. Schonewille H, van de Watering LMG, Loomans DSE et al. Red blood cell alloantibodies after transfusion: factors influencing incidence and specificity. *Transfusion*. 2006;46:250–56.
76. Sokol RJ, Stamps R, Booker DJ et al. Posttransplant immune-mediated hemolysis. *Transfusion*. 2002;42:198–204.
77. Young PP, Uziebla A, Trulock E et al. Autoantibody formation after alloimmunization: are blood transfusions a risk factor for autoimmune hemolytic anemia? *Transfusion*. 2004;44:67–72.
78. Zumberg MS, Proctor JL, Lottenberg R et al. Autoantibody formation in the alloimmunized red blood cell recipient. *Arch Intern Med*. 2001;161:285–90.
79. Moise KJ. Red blood cell alloimmunization in pregnancy. *Semin Hematol*. 2005;42:169–78.
80. Drabik-Clary K, Reddy VVB, Benjamin WH et al. Case report: severe hemolytic disease of the newborn in a Group B African-American infant delivered by a Group O mother. *Ann Clin Lab Sci*. 2006;36:205–7.
81. Oepkes D, Seaward PG, Vandenbussche F et al. Doppler ultrasonography versus amniocentesis to predict fetal anemia. *N Engl J Med*. 2006;355:156–64.
82. Geaghan SM. Diagnostic laboratory technologies for the fetus and neonate with isoimmunization. *Semin Perinatol*. 2011;35:148–54.
83. Illanes S, Soothill P. Management of red cell alloimmunisation in pregnancy: the non-invasive monitoring of the disease. *Prenat Diagn*. 2010;30:668–73.
84. Daniels G, Finning K, Martin P et al. Fetal blood group genotyping: present and future. *Ann NY Acad Sci*. 2006;1075:88–95.
85. Dinesh D. Review of positive direct antiglobulin tests found on cord blood sampling. *J Pediatr Child Health*. 2005;41:504–7.
86. Blanco E, Johnston DL. Neutropenia in infants with hemolytic disease of the newborn. *Pediatr Blood Cancer*. 2012;58:950–52.
87. Sarici SU, Yurdakokk M, Serdar MA et al. An early (sixth-hour) serum bilirubin measurement is useful in predicting the development of significant hyperbilirubinemia and severe ABO hemolytic disease in a selective high-risk population of newborns with ABO incompatibility. *Pediatrics*. 2002;109:e53.
88. Dillon A, Chaudhari T, Crispin P et al. Has anti-D prophylaxis increased the rate of positive direct antiglobulin test results and can the direct antiglobulin test predict need for phototherapy in Rh/ABO incompatibility? *J Paediatr Child Health*. 2011;47:40–43.



20 Hemolytic Anemia: Nonimmune Defects

LINDA A. SMITH, PhD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *microangiopathic hemolytic anemia (MAHA)* and list several associated disorders and the age group most commonly affected.
2. Describe the general morphology and hematologic values associated with MAHA and criteria that distinguish disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), and hemolytic uremic syndrome (HUS).
3. Recognize the characteristic erythrocyte morphology of MAHA on a stained blood film.
4. Identify organisms that can cause erythrocyte hemolysis.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Summarize the general pathophysiology for MAHA.
2. Compare and contrast the clinical findings, underlying cause, treatment, and characteristic findings for erythrocytes, platelet count, and coagulation tests for each of the following types of MAHA:
   a. Hemolytic uremic syndrome (HUS)
   b. Thrombotic thrombocytopenic purpura (TTP)
   c. Disseminated intravascular coagulation (DIC)
3. Define *exercise-induced hemoglobinuria*.
4. Given a set of data and clinical history, determine whether MAHA is a probable diagnosis, identify the possible etiology, and propose follow-up tests that should be performed.
5. Compare the cause of hemolysis by the following infectious agents:
   a. *Plasmodium* parasites (malaria)
   b. *Babesia* parasites
   c. *Bartonella* bacteria
   d. *Clostridium* bacteria

Chapter Outline

Key Terms

Cryosupernatant
Disseminated intravascular coagulation (DIC)
Fresh frozen plasma (FFP)
Hemolysis, elevated liver enzymes and low platelet (HELLP) syndrome
Hemolytic uremic syndrome (HUS)
Microangiopathic hemolytic anemia (MAHA)
Plasma exchange
Thrombotic thrombocytopenic purpura (TTP)
von Willebrand factor (VWF)

Background Basics

The information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review the following concepts:

Level I

- Describe the normal production, life span, and destruction of the erythrocyte. (Chapter 5)
- List the reference intervals for basic adult hematology parameters. (Chapters 1, 10)
- Identify sources of defects that lead to hemolytic anemia. (Chapter 11)
- Review the immune hemolytic anemias, and describe how they differ from other hemolytic anemias. (Chapter 19)
- Identify the intrinsic hemolytic anemias, and describe how they differ from extrinsic hemolytic anemias. (Chapter 11)

Level II

- Describe the structure and function of the major proteins of the erythrocyte membrane. (Chapter 5)
- Identify the key tests that can be used in diagnosis of anemia, and identify clinical signs of anemia. (Chapter 11)
- Describe the different nonimmune mechanisms of hemolysis and how they are detected. (Chapters 5, 11, 17, 18)
- Identify the laboratory tests that differentiate immune from nonimmune anemia. (Chapters 11, 19)

CASE STUDY

We will refer to this case study throughout the chapter.

Mai, a 35-year-old woman, saw her physician. She complained of weakness, low-grade fever, periods of forgetfulness, and memory loss for the last week or so. She denied any viral illness before the onset of symptoms. She was on oral contraceptives but was not taking any other drugs. Her initial laboratory tests showed:

Hb	6 g/dL (60g/L)
Hct	18% (0.18 L/L)
WBC	8.9×10^9/L

Consider reflex testing that could be helpful in identifying the cause of the anemia.

OVERVIEW

This chapter deals with the mechanisms of hemolysis not included in the chapters on membrane defects (Chapter 17), metabolic deficiencies (Chapter 18), or immune mechanisms (Chapter 19) and includes microangiopathic hemolytic anemias (MAHAs). Hemolytic uremic syndrome (HUS), an MAHA, is discussed in detail including disease association, pathophysiology, and clinical and laboratory findings. It also provides a brief overview of thrombotic thrombocytopenic purpura (TTP), which is discussed in detail in Chapter 35. Uncommon causes of hemolysis such as hypertension, mechanical heart devices, burns, exercise, and infectious agents also are discussed.

INTRODUCTION

Erythrocytes that have normal hemoglobin structure, enzymes, and membranes can be prematurely destroyed by factors extrinsic to the cell. This destruction can be immune mediated via antibodies and/or complement (Chapter 19). However nonimmune factors also can cause either extravascular or intravascular hemolysis, depending on the type and extent of injury to the erythrocyte. This chapter discusses nonimmune causes that lead to premature erythrocyte destruction. Erythrocytes can undergo traumatic physical injury in the peripheral circulation, resulting in the presence of schistocytes in the peripheral blood. Contact with fibrin strands or platelet aggregates in the microcirculation or with foreign surfaces such as artificial heart valves commonly induce such damage. There are other causes of injury, including Shiga toxin from organisms such as *Escherichia coli* 0157:H7. Infectious agents such as *Plasmodium sp.* and *Babesia sp.* can cause injury to the erythrocytes during their intracellular life cycle. Some drugs and chemicals can cause membrane oxidant injury, leading to intravascular hemolysis or removal of the damaged cell by the spleen. (Table 20-1 ★).

HEMOLYTIC ANEMIA CAUSED BY PHYSICAL INJURY TO THE ERYTHROCYTE

Intravascular and/or extravascular hemolysis and striking abnormal shapes of the circulating peripheral blood erythrocytes, including fragments (schistocytes) and helmet cells characterize hemolytic anemia caused by traumatic physical injury to the erythrocytes in the vascular circulation.

★ **TABLE 20-1** Hemolytic Anemias Caused by Nonimmune Antagonists in the Erythrocyte Environment

Category	Antagonist	Mode of Hemolysis
Microangiopathic hemolytic anemia (HUS, TTP, DIC)	Thrombi in microcirculation	Physical damage to erythrocytes by microthrombi
Malignant hypertension	Unknown	Physical damage to erythrocytes
Other physical trauma		
Exercise-induced hemoglobinuria	External force	Fragmentation of erythrocytes from excessive external force as they pass through microcapillaries
Thermal injury	Heat	Thermal damage to erythrocyte membrane proteins
Traumatic cardiac	Physical stress	Erythrocyte fragmentation
Infectious agents	*Plasmodium* sp.	Direct parasitization of erythrocyte; hypersplenism; acute intravascular hemolysis (*P. falciparum* infection)
	Babesia sp.	Invasion of the erythrocyte and cell lysis
	Bartonella sp.	Invasion of the erythrocyte and cell lysis
	Clostridium sp.	Hemolytic toxins
Animal venoms	Snake bites	Mechanical cell damage due to DIC
	Spider bites	Venom
Chemicals and drugs	Water	Osmotic lysis
	Oxidants	Hemoglobin denaturation
	Lead	Erythrocyte membrane damage

Microangiopathic Hemolytic Anemia

Microangiopathic hemolytic anemia (MAHA), sometimes referred to as *thrombotic microangiopathic anemia (TMA)*, is an inclusive term referring to a hemolytic process caused by microcirculatory lesions. Damage to the endothelial lining of the small vessels results in deposits of fibrin (which sometimes includes platelets) within the vessel. As the erythrocytes are forced through the fibrin strands, the membrane can be sliced open. Sometimes the erythrocyte membrane reseals itself, leading to abnormal erythrocyte shapes that are noted as schistocytes and keratocytes on the peripheral blood smear (Chapter 10). These damaged cells are often removed in the spleen (extravascular hemolysis). Severely damaged erythrocytes can be destroyed intravascularly. Depending on the underlying pathology, leukocytes can be increased and platelets can be decreased. Evidence of intravascular coagulation (formation of fibrin within the blood vessels) and fibrinolysis (breakdown of fibrin) could be present. The plasma concentration of markers of hemolysis (bilirubin and haptoglobin) varies depending on the type and extent of hemolysis (Chapter 11).

The underlying diseases and conditions responsible for microangiopathic hemolytic anemia include hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), malignant hypertension, disseminated cancer, and pregnancy (eclampsia, preeclampsia). A severe form of preeclampsia characterized by **hemolysis, elevated liver enzymes and low platelet counts (HELLP) syndrome** can also cause MAHA. HUS and TTP can present with similar initial clinical symptoms, but the underlying etiology, the age group affected, and the target organ(s) differ (Table 20-2 ★).

☑ CHECKPOINT 20-1

What abnormal erythrocytes characterize microangiopathic hemolytic anemia? How are these cells formed?

CASE STUDY (continued from page 373)

Mai's peripheral blood smear showed moderate schistocytes and polychromasia.

1. What are some conditions that result in the presence of schistocytes?

Hemolytic Uremic Syndrome

Hemolytic uremic syndrome (HUS) is a multisystem disorder first described in the mid-1950s. It is characterized by a triad of clinical findings:

- hemolytic anemia with erythrocyte fragmentation
- thrombocytopenia
- acute nephropathy, which can include renal failure

Most individuals recover renal function.[1–3] In some cases, there can also be evidence of mild neurologic problems although only a small percentage develop severe symptoms.

★ **TABLE 20-2** Comparison of Characteristics Associated with HUS and TTP

TTP	HUS
Adults ages 20–50	Children <5 years old
Hemolytic anemia with red cell fragmentation	Hemolytic anemia with red cell fragmentation
Renal dysfunction (mild to moderate)	Acute renal failure
Thrombocytopenia	Thrombocytopenia
Severe CNS symptoms	Mild CNS symptoms
Fever	

★ **TABLE 20-3** Types of HUS and Associated Conditions That Can Precipitate HUS

Type of HUS	Associated Condition
Diarrhea related (classic)	*Escherichia coli* O157:H7
D+ HUS	Other serotypes of *E. coli*
	Shigella dysenteriae serotype I
Nondiarrhea-related D− HUS	Postinfectious
	Streptococcus pneumoniae
	Viral infections
	Immunosuppression related
	Chemotherapy/cytotoxic drugs
	Renal and bone marrow transplantation
	Pregnancy or oral contraceptive related

HUS can be subdivided into two groups (D+ HUS and D− HUS) based on the presence or absence of a bloody diarrheal prodrome (Table 20-3 ■). The diarrhea-associated (D+ HUS) is the most common form and represents about 90% of cases. Onset of D+ HUS tends to occur in children between the ages of 6 months and 10 years; however, most cases are seen in children up to the age of 5 years. It is also considered the most common cause of acute renal failure in children and can lead to chronic renal insufficiency (Table 20-2). Adult-onset D+ HUS, which is infrequent, occurs in those over 16 years of age. The elderly are at highest risk for severe disease.

D+ HUS

D+ HUS is characterized by a bloody diarrhea with >90% of the cases associated with gastrointestinal infections by specific serotypes of *Escherichia coli*, which produce Shiga toxin (Stx). Most other cases are associated with infection by *Shigella dysenteriae* Type 1. The incubation period from infection to onset of diarrhea is usually 5–6 days but can range from 1–14 days. However, only about 15% of individuals who are infected with Shiga toxin-producing *E. coli* develop HUS.[3–7]

In the United States, the most common serotype of Shiga toxin-producing *E. coli (STEC)*, or enterohemorrhagic *E. coli (EHEC)*, is *E. coli* O157:H7. In other countries different serotypes are linked to D+ HUS.[3,7] A recent outbreak in adults in Germany was associated with infection by *E. coli* O104:H4 that had acquired a *Stx* gene.[8] The causative organism is not part of the normal flora of humans but is found in the gastrointestinal tract of a small percentage of cattle. The majority of human infections have been traced to the ingestion of incompletely cooked beef contaminated with the organism, but the organism can also be transmitted by the fecal-oral route (inadequate hand washing).[4,9]

Other factors such as elevated neutrophil counts, increased C-reactive protein (CRP), and fever are associated with an increased risk for developing HUS.[10–12] The use of antibiotics or antimotility drugs for treating individuals with diarrhea from *E. coli* O157:H7 has also been associated with increased risk for developing HUS.[13–15]

D− HUS

The second category of HUS is that of nondiarrhea-associated D− HUS, also referred to as *atypical HUS (aHUS)* or *STEC negative HUS (STEC− HUS)*. This condition has been reported in both children and adults and is the type that is more likely to recur.[16–18] aHUS has been attributed to various causes[17] (Table 20-3). It has been associated with connective tissue diseases such as systemic lupus erythematosus and lupus anticoagulant, as well as some types of cancer (especially stomach, colon, and breast) and diabetes.[17,19] The disease has been reported in young women with complications of pregnancy, after normal childbirth, or with the use of oral contraceptives.[20,21] In recent years, aHUS also has been associated with immunosuppressive therapy used in solid organ and bone marrow transplantation.[22–25] aHUS has been linked to invasive *Streptococcus pneumoniae* infections as well as viral infections such as Epstein-Barr virus, human immunodeficiency virus, and cytomegalovirus.[26–29] *S. pneumoniae* is the organism most commonly linked to aHUS. In most cases, the individual had an invasive infection such as pneumonia or bacteremia and the causative organism belonged to a serotype not included in pneumococcal vaccines.[30]

Many drugs including antiplatelet drugs and antineoplastic agents have been reported to cause rare cases of aHUS.[22, 31] HUS secondary to other diseases has a higher risk of recurrence and a lower survival rate than cases that are associated with colitis or have no identifiable trigger (primary).[31]

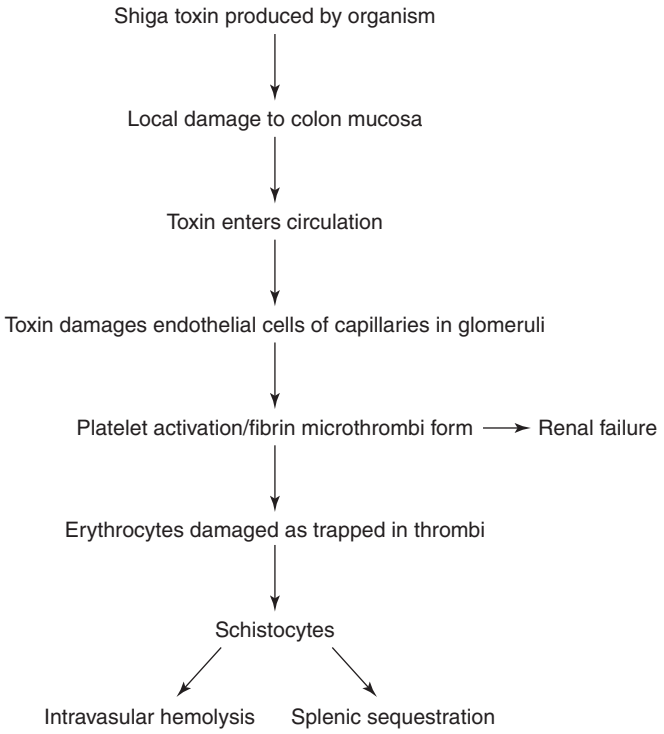
In recent years, mutations of several complement regulatory proteins have been associated with familial or inherited aHUS; in these cases, episodes of HUS often recur. Almost 50% are mutations related to three regulatory proteins associated with the alternate complement pathway.[18,32–36] These proteins, which function to control amplification of the pathway by C3bBb, are factor H, membrane cofactor protein (MCP, CD46), and factor I. An autoantibody against factor H has also been implicated in some acquired cases of aHUS.[37,38] The impaired regulation of the complement cascade leads to increased endothelial deposition of C3, activation of the membrane attack complex (MAC), glomerular endothelial cell destruction, and microvascular thrombosis.

☑ CHECKPOINT 20-2

What are the two types of HUS, and what organisms or diseases are most commonly associated with each type?

Pathophysiology

More than 70% of the cases of HUS have been associated with damage to the renal glomerular capillary endothelium by the Shiga toxin produced by *E. coli* O157:H7 and *S. dysenteriae* Type 1.[3,7] Once the organism enters the human gastrointestinal tract, it colonizes and forms an attachment lesion and begins to secrete virulence factors including Stx.[4] The organism's toxin is absorbed into the circulation through damaged gastrointestinal tissue and onto the surface of neutrophils[39] (Figure 20-1 ■). Neutrophils then deliver the Stx to the kidney. This toxin has a predilection for endothelial cells of the microvasculature of the glomerulus and exerts a direct toxic effect when bound to the endothelial cells. The toxin's B subunits bind to plasma membrane Gb3 receptors while the A subunit inhibits ribosomal protein synthesis, leading to cell death.[1,4] Infiltrates of inflammatory cells and lipopolysaccharides and the production of cytokines such as IL-8 and tumor necrosis factor-α contribute to the cytotoxic damage in glomerular and renal tubular cells.[40] Endothelial damage leads to the release of prothrombotic-, vasoactive-, and platelet-aggregating



■ **FIGURE 20-1** A possible mechanism for damage by Shiga-like toxin of *Escherichia coli* O157:H7.

substances that cause platelet activation with the subsequent formation of thrombi.[41,42] Although damage primarily occurs in the renal microvasculature, other organ systems (central nervous system, heart, liver) can be affected. The resulting thrombotic microangiopathy that traps erythrocytes and causes fragmentation is responsible for the schistocytes commonly seen in HUS.

In *S. pneumonia* infections (*pneumococcal HUS*), the bacterial enzyme neuraminidase is responsible for the capillary damage. Neuraminidase cleaves cell membrane glycoproteins and glycolipids, facilitating tissue invasion by the bacteria and exposing the normally hidden T-antigen (Thomsen-Friedenreich antigen) on capillary walls, platelets, and erythrocytes. Naturally occurring anti-T antibodies cause agglutination of cells and platelets leading to thrombosis in the small vessels.[27] Some evidence suggests that increased C3b deposition on cells may lead to cell destruction via MAC.[40] Catabolic enzymes (especially leukocyte elastase) and oxidative products released from the granules of activated neutrophils have been implicated in causing additional endothelial damage.[42]

☑ CHECKPOINT 20-3

Explain how infection with *E. coli* O157:H7 results in intravascular hemolysis.

Clinical Findings

D+ HUS occurs in previously healthy children with the highest incidence in the first year of life. The onset is acute with sudden pallor, abdominal pain, vomiting, foul-smelling and bloody diarrhea, and macroscopic hematuria. Other symptoms include a low-grade fever, hypertension, petechiae, bruising, and jaundice. The most important and/or serious complication of HUS is acute renal failure, which can lead to chronic renal insufficiency in some children. The duration of oliguria and anuria is variable. Organs other than the kidney can be affected as well.

Regardless the organ affected, the pathology is the same (i.e., thrombosis of the microcirculation). Central nervous system symptoms can result directly from microangiopathy of the central nervous system or from resulting hypertension. Lethargy and minor seizures are the most common symptoms. Hepatomegaly can be present; splenomegaly is less common. Hyperglycemia is common in children because of pancreatic damage secondary to HUS.

Laboratory Findings

A moderate to severe normocytic, normochromic anemia is typical with hemoglobin levels as low as 3–4 g/dL (median values 7–9 g/dL) (Table 20-4 ★). The peripheral blood smear shows fragmented and deformed cells (schistocytes, burr cells, helmet cells, spherocytes) with the degree of anemia correlated directly with the degree of morphologic change (Figure 20-2 ■). Polychromasia and an occasional nucleated erythrocyte can be seen. A leukocytosis with a shift to the left is common. Platelet counts vary from low normal to markedly decreased with a median value of 50 $\times$ 10^9/L. The duration of thrombocytopenia is 1–2 weeks.

Hemoglobinemia with an increase in total serum bilirubin (2–3 mg/dL) and a decrease in serum haptoglobin reflects chronic intravascular hemolysis. Serum lactate dehydrogenase (LD) is markedly elevated, and cardiac enzymes can be elevated because of myocardial damage.[2,6] Blood urea nitrogen (BUN) and creatinine levels are increased, reflecting renal damage. Metabolic acidosis, hyponatremia, and hypokalemia are common. Screening for complement abnormalities (CH50 assay, C3 and C4 levels, complement factor H [CFH] and complement factor I [CFI] levels) can be indicated in patients with symptoms of aHUS.[18] The CH50 is a test of total complement activity and is reported as the reciprocal of the serum dilution that lyses 50% of a red cell suspension.

★ **TABLE 20-4** Laboratory Findings in HUS and TTP

Evidence of hemolysis	Decreased hemoglobin/hematocrit
	Increased reticulocytes/polychromasia
	Thrombocytopenia
	Leukocytosis with shift to the left
	Presence of schistocytes
Evidence of intravascular hemolysis	Hemoglobinemia
	Hemoglobinuria
	Decreased haptoglobin
	Increased total and unconjugated serum bilirubin
	Increased lactic dehydrogenase (LD)
Evidence of thrombotic microangiopathy	Thrombocytopenia
	Fibrin degradation products (normal to slightly increased); D-dimer increased
	PT and APTT (normal to slightly abnormal)
	Factors I, V, VIII (normal to increased)

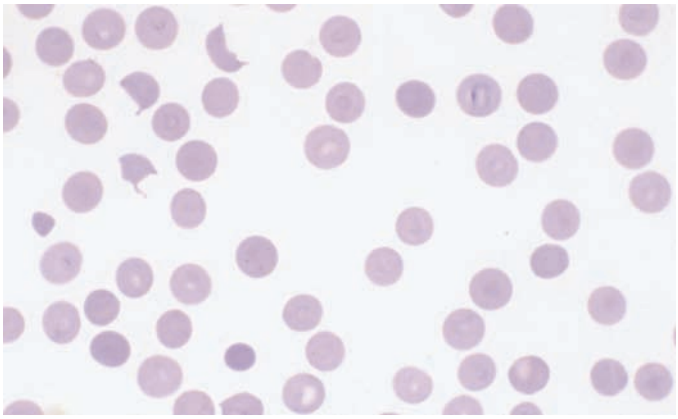


FIGURE 20-2 A peripheral blood smear from a patient with hemolytic uremic syndrome. The platelets are markedly decreased. Schistocytes and spherocytes are present (Wright-Giemsa stain, 1000× magnification).

Screening tests for coagulation abnormalities include the prothrombin time (PT) and activated partial thromboplastin time (APTT). Many cases of HUS exhibit no detectable consumption of coagulation factors.[42] The PT can be normal or slightly prolonged, but the APTT is usually normal. Although fibrin-degradation products (fragments of fibrin produced by plasmin degradation of fibrin) and D-dimer are elevated, disseminated intravascular coagulation (DIC) is rare. In DIC (see the later section "Disseminated Intravascular Coagulation"), coagulation screening tests are abnormal. The direct and indirect antiglobulin tests are usually negative, reflecting a nonimmune pathology.

Urinalysis results show moderate to massive amounts of protein (1–2 g/24 hours to 10 g/24 hours), gross and microscopic hematuria, increased numbers of neutrophils (pyuria), and casts (hyaline, granular, renal epithelial), reflecting the damage to the glomerulus. The presence of hemosiderin in the urine sediment reflects chronic intravascular hemolysis (Chapter 11).

☑ CHECKPOINT 20-4

What are the typical erythrocyte morphology and coagulation test results in children with HUS?

Therapy

Mild to moderately severe cases of D+ HUS have the best prognosis for recovery (>80%). With improvement in early diagnosis and supportive care, especially during oliguric or anuric phases, the mortality of the disease has been reduced to 5–15%. Supportive care includes close observation, blood transfusion if necessary, control of electrolyte and water imbalances, control of hypertension, and peritoneal dialysis in anuria. Platelet transfusions are not recommended because they can exacerbate the thrombotic process but can be required in some patients with excessive bleeding. The beneficial use of fresh frozen **plasma exchange** (removal of patient plasma and replacement with donor plasma) has not been shown to be efficacious in patients with D+ HUS. In contrast, patients with aHUS, especially that caused by complement dysregulation, can benefit from plasma exchange or plasma infusions. Plasma infusions are contraindicated in patients with HUS who have a positive direct antiglobulin test or who are infected with *S. pneumoniae* because of the presence of naturally occurring anti-T in the infused plasma.[27]

A potentially promising preventative measure in individuals infected with Shiga toxin-producing *E. coli* is the use of monoclonal antibodies against Shiga toxin to provide passive immunity.[43,44] There is only a small window of time (3–5 days) from onset of diarrhea in which treatment can be effective, so detection of the toxin in stool or blood by flow cytometry must be performed.[45,46]

The drug eculizamab is a recombinant, monoclonal anti-C5 antibody. Originally used to treat paroxysmal nocturnal hemoglobinuria, it has proved to be effective in aHUS. The drug binds to C5 and prevents cleavage into C5a and C5b, thus preventing progression to the membrane attack complex (MAC).[47]

Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura (TTP) is another relatively uncommon disorder in which platelet aggregation on the microvascular endothelium results in serious complications. In most cases, it is an acute disorder that affects young adults (ages 20–50 with a peak incidence in the third decade). TTP occurs more frequently in females than males. TTP can be congenital or acquired (nonidiopathic or idiopathic).

Various clinical events have been identified as possible precipitating factors in acquired TTP (Table 20-5 ★). Infections are the most common precipitating factor (40%) followed by pregnancy (10–25%). Without treatment, TTP has a mortality rate in excess of 90% because of multiorgan failure. The disorder is discussed in detail in Chapter 35, but an overview comparison with HUS in this chapter highlights several key findings.

★ **TABLE 20-5** Some Reported Clinical Conditions That Can Be Precipitating Factors in TTP

Conditions	Examples
Infections	Bacterial—enteric organisms (*Shigella* sp., *E. coli*, *Salmonella* sp., *Campylobacter* sp., *Yersinia* sp.)
	Bacterial—other (*Streptococcus pneumoniae*, *Legionella* sp., *Mycoplasma* sp.)
	Viral (HIV, EBV, influenza, herpes simplex)
Drugs	Antimicrobials—penicillin
	Ticlopidine Chemotherapeutic agents
Connective tissue diseases	Systemic lupus erythematosus
	Rheumatoid arthritis
	Ankylosing spondylitis
	Sjögren's syndrome
Miscellaneous	Bee sting
	Dog bite
	Carbon monoxide poisoning
Pregnancy or oral-contraceptive related	
Lymphomas and carcinomas	

Pathophysiology

TTP is characterized by microthrombi composed of platelets and unusually large forms of **von Willebrand factor (VWF)** (Chapters 31, 32) that occlude capillaries and arterioles in a number of organs including the kidneys, heart, brain, and pancreas. (VWF is a plasma protein needed for platelets to adhere to collagen.) Although these thrombi contain platelets and sometimes immunoglobin and complement, there is little fibrin, inflammation, or subendothelial exposure as in disseminated intravascular coagulation (DIC).[48–50]

Ultralarge multimers of VWF normally are cleaved into smaller forms by the protease ADAMTS13 (a disintegrin-like and metalloprotease domain with thrombospondin type motifs) (Chapter 34). It is now known that a deficiency in ADAMTS13 is the cause of TTP.[51–54] As a result of an ADAMTS13 deficiency, these ultralarge VWF multimers remain attached to the endothelial cells and adhere to platelets, inducing platelet aggregation and formation of platelet thrombi. As erythrocytes are forced through the thrombi, fragmentation occurs. The familial form of TTP occurs because of a mutation in the *ADAMTS13* gene, resulting in a deficient/dysfunctional enzyme.[54] The acquired type is caused by autoantibodies against ADAMTS13, which block its activity.

Clinical Findings

The clinical aspects of TTP are similar to those of HUS except that TTP occurs most often in young adults and involves more organ systems. Neurologic symptoms are more prominent, renal dysfunction is less severe, and the mortality rate is higher than in HUS (Table 20-2). Symptoms can be eliminated with early treatment, although some patients recovering from TTP can have permanent manifestations of renal damage and require dialysis.

Laboratory Findings

Typical laboratory results are shown in Table 20-4. The hemoglobin is usually <10.5 g/dL (105 g/L; average 80–90 g/L). The mean cell volume (MCV) is variable, either normal or decreased if there is marked erythrocyte fragmentation or increased in the presence of reticulocytosis. The mean corpuscular hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) are normal. Nucleated erythrocytes can be found in the peripheral blood, reflecting the bone marrow response to hemolysis. The most striking blood finding is the abundance of schistocytes (generally $>1\%$).[55] Leukocytosis with counts of $>20 \times 10^9$/L occurs in 50% of patients and is usually accompanied by a shift to the left. Thrombocytopenia is often severe ($8–44 \times 10^9$/L) because of consumption of platelets in the formation of microthrombi. Megakaryocytes are abundant in the bone marrow, reflecting an increased hematopoietic response to the consumption of platelets.

Coagulation tests are usually normal or only mildly disturbed in TTP, which helps differentiate TTP from DIC in which an increase in D-dimer as well as a prolonged PT, APTT, and thrombin time occur (Chapter 35).[56]

Hemoglobinemia, hemoglobinuria, decreased haptoglobin levels, and increased total and unconjugated serum bilirubin are direct evidence of intravascular hemolysis.

Therapy

Studies have shown that plasma exchange with **fresh frozen plasma (FFP)** can be effective in providing the needed ADAMTS13 protease and removing autoantibody (in acquired TTP).[57–62] **Cryosupernatant**, which lacks the large VWF multimers present in FFP yet still contains the needed VWF cleaving protease, can be used. Treatment options include the monoclonal antibody rituximab (anti-CD20), antiplatelet or platelet-inhibiting agents, intravenous administration of steroids, or combinations of corticosteroids and plasma.[63,64]

☑ CHECKPOINT 20-5

How does the clinical presentation of TTP differ from that of HUS? How is it similar?

CASE STUDY (continued from page 374)

As Mai was questioned further, she indicated that she had noticed a large number of bruises on her extremities. Her platelet count was 31×10^9/L. She had a 2.5% reticulocyte count.

2. What is the significance of these results?
3. Why might the clinician order coagulation tests?

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is a complex thrombohemorrhagic condition in which the normal coagulation process is altered by an underlying condition. The more common conditions that precipitate DIC include bacterial sepsis, neoplasms, immunologic disorders, or trauma[65] (Table 20-6 ★). DIC can be initiated by damage to the endothelial lining of vessels, which causes release of thromboplastic substances that activate the coagulation mechanism. As a result, platelet activation and aggregation and activation of plasma procoagulant proteins lead to deposition of fibrin and formation of microthrombi in the microvasculature. As erythrocytes become entangled in the fibrin meshwork in the capillaries (clothesline effect), they fragment to form schistocytes. Complications that result include thrombotic occlusion of vessels, bleeding because of consumption of coagulation proteins, and ultimately organ failure. Hemolysis is not usually severe, but the effects of the consumptive coagulopathy can cause severe thrombocytopenia and serious bleeding complications.

★ **TABLE 20-6** Causes of Disseminated Intravascular Coagulation (DIC)

Bacterial sepsis	Endotoxins
	Exotoxins
Neoplasm	Solid tumors
	Myeloproliferative disorders
Serious trauma	——
Immunologic disorders	Hemolytic transfusion reactions
	Transplant rejection
Miscellaneous	Venom—snake or insect
	Drugs

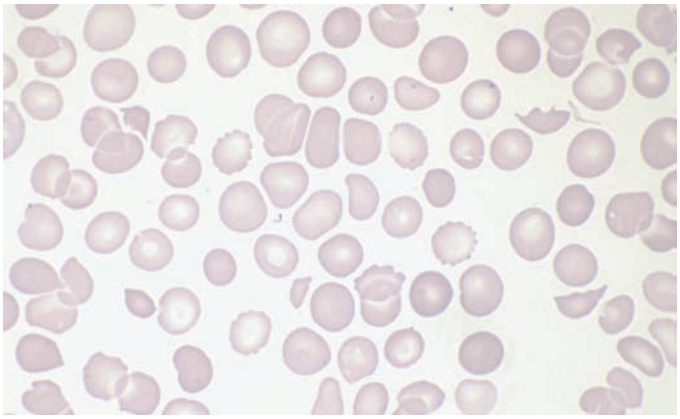


FIGURE 20-3 Peripheral blood from patient with disseminated intravascular coagulation. Notice the schistocytes and thrombocytopenia (Wright stain, 1000× magnification).

The typical findings on the blood smear include the presence of schistocytes and thrombocytopenia (Figure 20-3 ■). The presence of schistocytes is not specific for DIC. However, the abnormal coagulation tests help distinguish this condition from others (TTP and HUS) that give a similar picture on a peripheral blood smear and increase diagnostic accuracy.[66,67] Abnormal coagulation tests include:

- Prolonged prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT)
- Elevated D-dimer test
- Decreased platelet count
- Increased fibrin degradation products (FDP)
- Decreased fibrinogen

In some cases, specific tests such as procalcitonin, an inflammatory biomarker, can be useful if septic DIC is suspected.[68]

Treatment can include erythrocyte and platelet transfusions as well as infusion of fresh frozen plasma or factor concentrates to replace coagulation factors. Most important, however, are the treatment and resolution of the underlying disorder responsible for the DIC. The etiology, diagnosis, and treatment of DIC is discussed further in Chapter 35.

☑ CHECKPOINT 20-6

Explain how DIC can be differentiated from TTP and HUS based on coagulation tests.

HELLP Syndrome

The HELLP syndrome is an obstetric complication characterized by hemolysis, elevated liver enzymes, and a low platelet count that usually develops prior to childbirth, often in the third trimester. A partial or incomplete form in which only 1 or 2 of the characteristic findings is present has also been described.[69] The etiology and pathogenesis are not well understood, but an association with pregnancy-induced hypertension and DIC is suggested.[70] As with TTP and HUS, the precipitating factor is often unknown, but the clinical aspects are characterized by capillary endothelial damage and intravascular platelet activation as well as microangiopathic hemolytic anemia.[69,70] Although a significant prevalence of HELLP occurs in patients with antiphospholipid syndrome, there seems to be no direct association with the presence of IgM or IgG anticardiolipin antibodies or anti-β2-glycoprotein-I antibodies.[71,72] Some experts consider HELLP to be a severe form of preeclampsia or eclampsia, which shares some of their characteristics such as hypertension, proteinuria, and thrombocytopenia, but it is distinguished from them by the presence of hemolysis and elevated liver enzymes. Severe cases can compromise fetal growth and survival. Approximately 10% of pregnancies with eclampsia develop HELLP syndrome with a mortality rate of about 1%.

The peripheral blood findings are similar to those found in TTP, HUS, and other microangiopathic conditions. Overall, however, the hemolysis and thrombocytopenia are less severe than those associated with TTP or HUS. Liver damage is due primarily to obstruction of hepatic sinusoids and can lead to subsequent hepatic hemorrhage or necrosis.[69] Microvascular fibrinlike deposits that resemble those in TTP/HUS are responsible for the presence of schistocytes. Laboratory markers are used to determine the presence of HELLP. The liver enzyme most frequently measured is aspartate aminotransferase (AST), and concentrations >70 IU/L are common. Increased total (>1.2 mg/dL) and/or unconjugated bilirubin, increased LD (generally >600 IU/L), and decreased haptoglobin can also be seen in HELLP.[69] Coagulation tests such as the PT and APTT are usually normal until late in the disease course.[69,73] The platelet count decreases (usually $<100 \times 10^9$/L) as a result of platelet consumption at the site of endothelial damage. Although DIC infrequently occurs as a complication of HELLP, patients with lower platelet counts ($<50{,}000 \times 10^9$/L) can be at increased risk.[69,74] Acute tubular necrosis with renal failure, hepatic rupture, and pulmonary edema can also occur as complications.

Corticosteroid therapy can be useful in controlling cell destruction and decreasing liver enzymes if the fetus cannot be delivered immediately. Plasma exchange is rarely used as a treatment. The use of cortisone dexamethasone has eliminated the need for platelet transfusions in most patients with platelet counts $<50 \times 10^9$/L.[73,75,76]

CASE STUDY *(continued from page 378)*

Mai's PT and APTT were slightly prolonged. The fibrinogen levels were slightly decreased.

4. What do these findings indicate about the underlying problem?

Malignant Hypertension

MAHA associated with malignant hypertension is characterized by a low platelet count and erythrocyte fragmentation. In addition, the presence of schistocytes, low platelet count, and increased LD has been used to predict renal insufficiency as well as recovery.[77] The mechanism of hemolysis is unknown. It has been suggested that it can be caused by endothelial injury, fibrinoid necrosis of arterioles, or deposition of

fibrin fed by thromboplastic substances released from membranes of lysed erythrocytes. One recent study showed that individuals with malignant hypertension had decreased levels of ADAMTS13 activity. This may be caused by release of increased amounts of VWF that occurs in hypertension because of endothelial stimulation.[78]

Other Conditions Associated with MAHA

Several reports have linked MAHA with diabetes. It is theorized that in these cases, the cholesterol to phospholipid ratio is altered, leading to a rigidity in the erythrocyte membrane.[20] Stem cell transplant recipients can show increased evidence of transplant-associated microangiopathy (TA-TAM) characterized by schistocytes, decreased platelet counts, decreased hemoglobin, and increased LD. Although the condition can resemble TTP, ADAMTS13 activity is >5%.[79] TA-TAM may represent a form of graft-vs-host disease (GVHD) and result from endothelial damage induced by donor cytotoxic T cells.[23,25]

Other Erythrocyte Physical Trauma Resulting in Hemolytic Anemia

Traumatic Cardiac Hemolytic Anemia

Hemolytic anemia is an uncommon complication following surgical insertion of prosthetic heart valves. Unlike the microangiopathic anemia seen with TTP or DIC, the platelet count usually does not significantly decrease. Excessive acceleration or turbulence of blood flow around the valve can fragment the erythrocytes as a result of "shear stress."[80,81] The term *Waring blender syndrome* has been used to describe this disorder because of the localized turbulent blood flow. Many erythrocyte fragments are apparent on the blood smear. The spleen removes some of the severely traumatized cells, but most of them undergo intravascular hemolysis. Newly designed prosthetic valves have helped decrease the shear stress and resulting erythrocyte fragmentation.[80] Hemolysis and erythrocyte fragmentation rarely have been associated with formation of large vegetations in patients with infective endocarditis.

Thermal Injury

Hemolytic anemia occurs within the first 24–46 hours after extensive thermal burns, and the degree of hemolysis depends on the percentage of body surface area burned. Hemolysis probably results from the direct effect of heat-causing protein denaturation of spectrin in the erythrocyte membrane. (If erythrocytes are heated to 48°C in vitro, spectrin degradation causes a loss of elasticity and deformability.) In addition, the fatty acid and lipoprotein metabolism in both plasma and erythrocytes are altered after burn injuries, which can contribute to abnormal erythrocyte morphology.[82] Peripheral blood smears show erythrocyte budding, schistocytes, and spherocytes. After 48 hours, signs of hemolysis such as hemoglobinuria and hemoglobinemia decrease. Thermal injury to erythrocytes also has occurred during hemodialysis when the dialysate is overheated.

Exercise-Induced Hemoglobinuria

Exercise-induced hemoglobinuria (sometimes described as *march hemoglobinuria*) describes a transient hemolysis occurring after strenuous exercise and often involves contact with a hard surface (e.g., running, tennis, marching). The hemolysis is probably because of physical injury to erythrocytes as they pass through the microvasculature. Plasma hemoglobin and haptoglobin levels indicate that the primary cause of the transient hemolysis is intravascular lysis because of "footstrike."[83] However, it is not seen in all individuals participating in these activities and is occasionally seen in other physical activities such as swimming, cycling, and rowing in which contact with a hard surface is limited. One recent study linked the presence of intravascular lysis to hand drumming.[84] In recent years, the role of exercise-induced oxidative stress and red cell age have been recognized as potential additional causes of lysis, especially in normally sedentary individuals who participate in strenuous exercise.[85] Increased osmotic fragility and decreased deformability leading to intravascular hemolysis were noted in these individuals.[86] In addition, changes in erythrocyte membrane proteins such as spectrin, especially in older cells, can increase susceptibility to extravascular hemolysis during strenuous exercise.

In contrast to the other hemolytic conditions discussed so far in this chapter, no erythrocyte fragments appear on the peripheral blood smear, but the hallmarks of intravascular hemolysis—hemoglobinemia and hemoglobinuria—can be present. The passage of reddish urine immediately after exercise and for several hours thereafter is usually the only complaint from affected individuals. Anemia is uncommon because <1% of the erythrocytes are hemolyzed during an episode. Individuals can present with slightly increased MCV as a result of increased reticulocytes.[83, 87] Iron deficiency can occur if exercise and hemolysis are frequent.

CASE STUDY *(continued from page 379)*

Mai's symptoms continued to become worse with frequent seizures, headaches, and dizziness. Her urinalysis results showed a 2+ protein and moderate blood. However, she had normal urinary volume.

5. Based on these results, what is the most likely condition associated with these clinical and laboratory results? Explain.
6. What therapy might be used?

HEMOLYTIC ANEMIAS CAUSED BY ANTAGONISTS IN THE BLOOD

Antagonists such as drugs or venoms and infectious organisms in the environment of the erythrocyte can cause premature destruction (Table 20-1). This hemolysis is precipitated by either injury to the erythrocyte membrane or to denaturation of hemoglobin.

Infectious Agents

Parasites and bacteria can infect erythrocytes and lead directly to their destruction. Alternatively, toxins produced by infectious agents can cause hemolysis.

Malarial Parasites

The anemia accompanying malaria is due directly and indirectly to the intracellular malarial parasites that spend part of their life cycle in the erythrocyte (Figure 20-4 ■). The anemia resulting from this

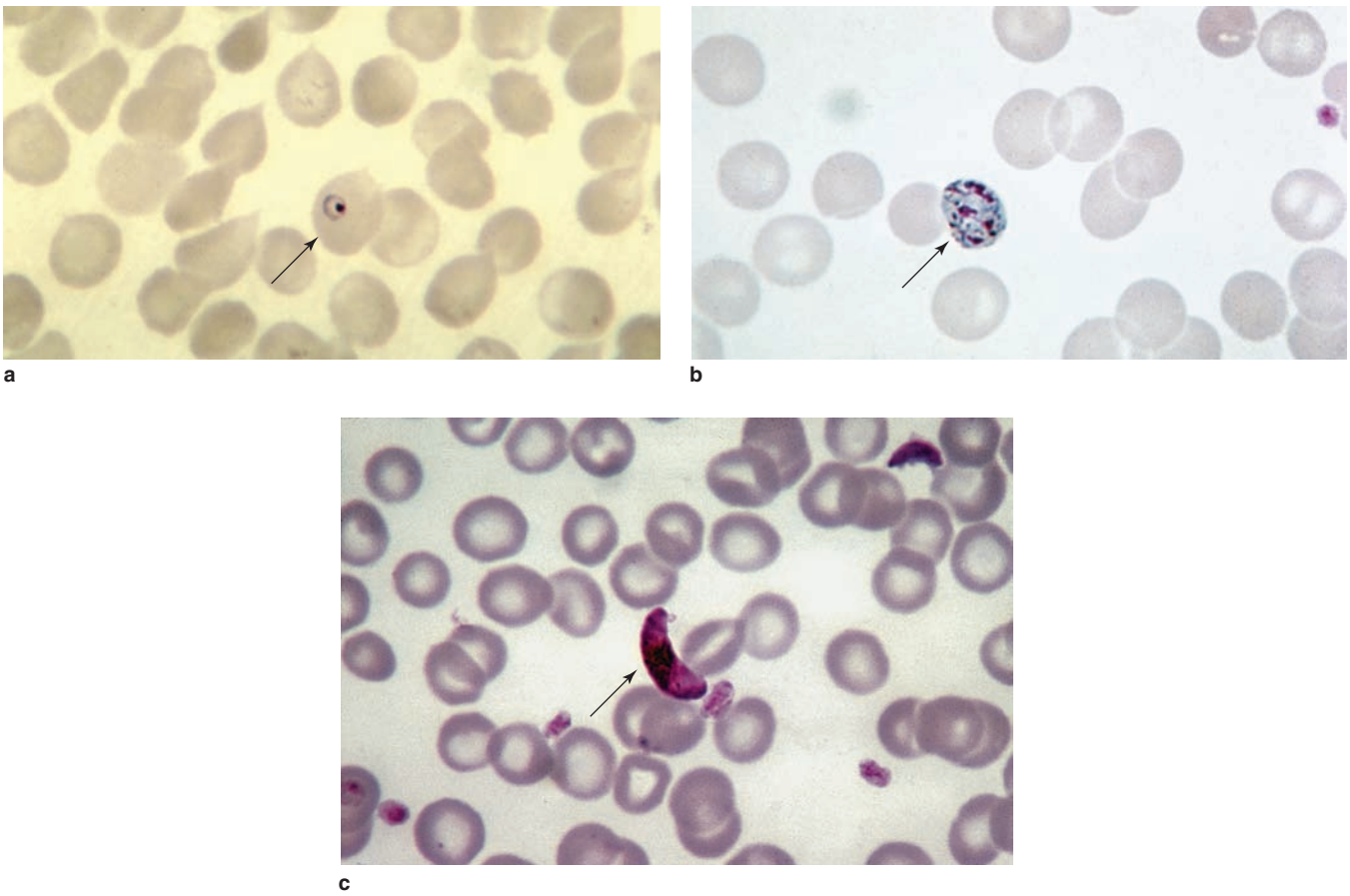


FIGURE 20-4 Peripheral blood smears from patients with malaria. **(a)** A ring form of malaria in the erythrocyte. **(b)** An immature schizont form of malaria in the erythrocyte. **(c)** A gametocyte of *Plasmodium falciparum* in the erythrocyte (Wright stain, 1000× magnification).

infection is usually a mild, normocytic normochromic anemia but can be severe in infection with *Plasmodium falciparum* because of the high levels of parasitemia. Thrombocytopenia can also be present. Diagnosis involves finding the life cycle stage within the erythrocyte on a peripheral blood smear. Infection with *P. falciparum*, a cause of severe anemia in children, can be accompanied by ineffective erythropoiesis and decreased reticulocytes. The hemoglobin in these cases can reach levels as low as 5 gm/dL.[88] In addition, poor diet, malnutrition, and decreased iron and folate stores contribute to the severity of the anemia. Exchange transfusions can be used in the severest cases to remove infected erythrocytes.[89]

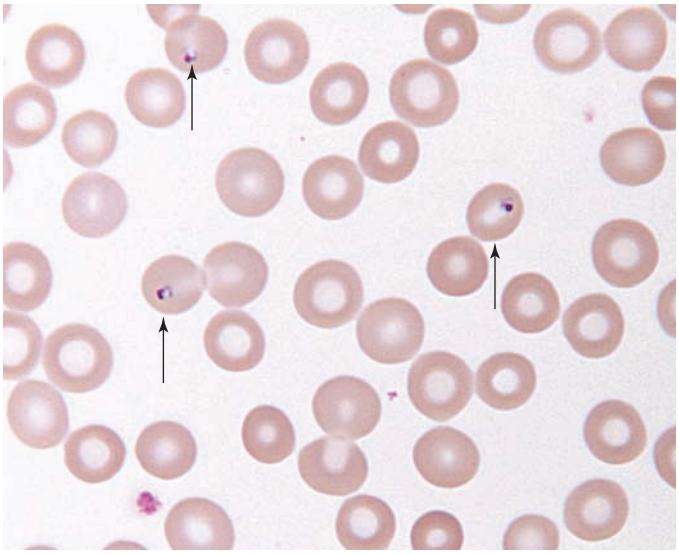
The release of the intraerythrocytic parasite from the cell results in the cell destruction. Also, the spleen can remove the entire parasitized cell, or splenic macrophages can pit the parasite from the erythrocyte, damaging the cell membrane. The resulting decreased deformability can lead to removal of the cell by the spleen. Anemia can also result from an immune-mediated process. Antimalarial antibodies react with malarial antigens on the erythrocyte membrane, resulting in removal of the sensitized cell by the splenic macrophages. In some cases, the concentration of complement regulatory proteins decrease, which can facilitate complement-mediated hemolysis.[90]

Blackwater fever, an uncommon complication of infection with *P. falciparum*, is characterized by massive acute intravascular hemolysis with hemoglobinemia, hemoglobinuria, methemalbuminemia, and hyperbilirubinemia. However, the parasitemia level is often low. The mechanism that precipitates this is unclear. One possible mechanism is the development of an autoantibody to the infected erythrocyte. Another is a direct reaction to the drug quinine or to repeated incomplete treatment with the drug. Quinine can act as a hapten to stimulate formation of a drug-dependent antibody that has complement-fixing ability.[88] In some cases, the direct antiglobulin test (DAT) can be positive with either monospecific anticomplement or anti-IgG. Use of synthetic quinine drugs has considerably decreased the frequency of this complication. It is possible that other antimalarial drugs including mefloquine could trigger this response.

Because intracellular parasites such as malaria often are initially detected in a peripheral blood smear, photomicrographs showing the morphology of the various species of malaria are available in the book's Companion Resources.

Babesiosis

Babesiosis, a protozoan infection of rodents and cattle, is most commonly transmitted to humans by the bite of a hard tick. However, the organism can be acquired transplacentally and through blood transfusions. In the United States, it occurs most frequently in the New England area but cases can be found in Virginia and the Midwest. The most



■ **FIGURE 20-5** Peripheral blood smear of a patient with babesiosis. Several infected erythrocytes are in the field (Wright stain, 1000× magnification).

common organism is *Babesia microti*. On the peripheral blood smear, the parasites appear as intracellular, pleomorphic, 1–5 mcM (μm) ringlike structures resembling ring-form trophozoites of *Plasmodium falciparum* (Figure 20-5 ■). Some can appear as doubles or tetrads in the form of a Maltese cross. Travel history, the absence of the characteristic banana-shaped gametocytes, and the lack of pigment help distinguish it from *P. falciparum*. Most infections are asymptomatic, but some cases present with a flulike syndrome. Generally there is 1–10% parasitemia.[91] Extravascular hemolysis can occur in a manner similar to that seen with malaria. A mild to moderate normocytic anemia as well as thrombocytopenia can be present. Other possible laboratory findings include increased reticulocyte count, liver enzymes, and bilirubin.[92] In a rare fulminating infection, severe anemia, intravascular hemolysis, and hemoglobinuria are seen. Complications associated with intravascular hemolysis include renal failure and disseminated intravascular coagulation. Individuals at greatest risk of complications are the elderly and those who are splenectomized or immunosuppressed. Patients who are splenectomized generally have a more severe clinical presentation and higher levels of parasitemia. In severe cases of hemolysis or renal complications, exchange transfusion can be indicated.

☑ CHECKPOINT 20-7

Why do malaria and babesiosis result in anemia?

Bartonellosis

Gram-negative bacterial organisms in the genus *Bartonella* are transmitted by blood-sucking arthropods or by direct inoculation by scratch or bite of a mammal. The organisms infect erythrocytes and endothelial cells. Infection by *Bartonella bacilliformis* is associated with Carrion's disease, which is currently restricted to Columbia, Peru, and Ecuador. Other *Bartonella* species infect erythrocytes but are not associated with hemolytic manifestations.[93] The disease is transmitted by the bite of a female sandfly and is biphasic with the acute phase characterized by an often fatal syndrome consisting of myalgia, high fever, and an acute, severe hemolytic anemia (Oroya fever).[93] Hepatosplenomegaly and jaundice can also be present. Changes in T lymphocyte counts and levels of cytokines such as IL-10 can also be associated with acute infection.[94] The disease can progress to coma and death within weeks and has a high mortality in untreated cases. If the patient survives, a chronic phase presents weeks to months later. The disease is characterized by a granulomatous reaction (verruga peruana) with the appearance of cutaneous hemangioma-like lesions that can contain bacteria, neutrophils, macrophages, endothelial cells, and deposits of immunoglobulins. The pleomorphic (coccobacillary) organisms are readily visualized as single, paired, or chained organisms on or within erythrocytes on Wright- or Giemsa-stained peripheral blood smears during the course of the disease. The organism releases several proteins including deformin that are responsible for inducing the pitting or invagination of the erythrocyte membrane. These structures, as well as other proteins such as hemolysins, can serve as entry portals for the bacteria and help explain the mechanism of cell destruction.[93,95]

Clostridium perfringens

C. perfringens is part of the normal flora of the gastrointestinal tract. Infection can present as a transient bacteremia or a life-threatening condition. It is one of the few organisms (along with *C. septicum* and *C. novyi*) that can cause a rapid, massive intravascular hemolysis, although this complication is very rare. Patients with neoplasms such as colon cancer or invasive tumors of the genitourinary tract are at highest risk.[96] The bacteria produce potent exotoxins that affect host cell membranes. The major hemolytic toxin (α-toxin) is a phospholipase C that hydrolyzes sphingomyelin and lecithin present in the erythrocyte membrane and leads to changes in membrane integrity. Fever, thrombocytopenia, neutrophilia, hemoglobinemia, and hemoglobinuria are present. Anuria or acute renal failure can develop as a result of the hemoglobinuria. Lysis of the erythrocyte or other cells can cause DIC. Bacterial neuraminidase can expose cryptic antigens such as Thomsen-Friedenrich (T antigen), which can react with nonimmune autoantibody. The peripheral blood smear shows many microspherocytes and few erythrocyte fragments.

Animal Venoms

Venoms injected by bees, wasps, spiders, and scorpions can cause hemolysis in some susceptible individuals. One of the more common bites that can result in hemolysis is that of the brown recluse spider (*Loxosceles reclusa*). The characteristic symptoms of envenomation are a localized lesion that shows signs of inflammation and a central thrombosis surrounded by ischemic areas. However, up to 15% of individuals, especially children, develop systemic symptoms including fever, jaundice, and intravascular hemolysis that can be severe.[97,98] Patients have leukocytosis, anemia, hematuria, thrombocytopenia, and increased levels of creatine phosphokinase (CK).[99] There are some reports of a positive DAT with complement on the erythrocyte after bites.[100] The mechanism of venom damage appears to involve sphingomyelinase D2, which cleaves glycophorin from the erythrocyte membrane. This decreases the structural integrity of the membrane and increases its sensitivity to complement-mediated lysis.[98,101] Although snake bites rarely

cause hemolysis directly, hemolysis can be the result of DIC or a venom-induced consumption coagulopathy (VICC) resulting from activation of the coagulation pathway.[102] VICC has no systemic microthrombi, but the D-dimer levels and activated PTT are elevated.

Chemicals and Drugs

Various chemicals and drugs have been identified as possible causes of erythrocyte hemolysis; many of these are dose dependent. In addition to erythrocyte hemolysis, chemicals and drugs can also produce methemoglobinemia and cyanosis, or in some instances bone marrow aplasia.

Hemoglobinemia and hemoglobinuria can occur as a result of osmotic lysis of erythrocytes when water enters the vascular system during transurethral resection or when inappropriate solutions are used during a blood transfusion.

Some drugs known to cause hemolysis in G6PD-deficient persons can also cause hemolysis in normal persons if the dose is sufficiently high. The hemolysis mechanism is similar to that in G6PD deficiency with hemoglobin denaturation and Heinz body formation because of strong oxidants.

Anemia associated with lead poisoning is usually classified with sideroblastic anemias because the pathophysiologic and hematologic findings are similar. Lead inhibits heme synthesis, causing an accumulation of iron within mitochondria (Chapter 12). However, lead also damages the erythrocyte membrane, which is manifested by an increase in osmotic fragility and mechanical fragility.

Summary

Mechanisms of nonimmune damage to the erythrocyte are varied. Hemolytic anemia caused by traumatic physical injury to the erythrocytes in the vascular circulation can be characterized by extravascular or intravascular hemolysis. Microangiopathic hemolytic anemia refers to a group of anemias caused by microcirculatory lesions that injure the erythrocytes, producing schistocytes. Of those discussed in this chapter, HUS and TTP are the more commonly encountered. HUS can be subdivided into two groups: D+ and D− based on the presence or absence of diarrhea. Classic D+ HUS is the most common and is mediated by the Shiga-like toxin of *E. coli* O157:H7. The toxin enters the gastrointestinal tract and damages the mucosa. Once it enters the circulation, it has a predilection for the endothelial cells of the glomerular microvasculature and exerts a toxic effect. Endothelial damage leads to release of prothrombotic substances that cause platelet activation and formation of thrombi. The thrombi trap erythrocytes and cause fragmentation of the cells. TTP is a disorder in which platelet aggregation and unusually large forms of von Willebrand factor (VWF) on the microvascular endothelium leads to formation of platelet thrombi that occlude capillaries and arterioles in multiple organs. The underlying cause of TTP is a deficiency in the ADAMTS13 protease that cleaves the ultralarge multimers of VWF into smaller forms. HELLP syndrome, another MAHA, is thought to be a severe form of preeclampsia. HELLP is characterized by hemolysis, elevated liver enzymes, and a low platelet count. Fibrin-like deposits are responsible for the presence of schistocytes. Antagonists in the blood such as venoms and infectious organisms can also cause hemolytic anemia. Intraerythrocytic parasitic infections with organisms such as *Plasmodium* sp. or *Babesia* sp. can cause hemolysis and anemia without the presence of schistocytes. In susceptible individuals, drugs or chemicals can also lead to hemolysis.

Review Questions

Level I

1. Which of the following results is associated with HUS and TTP? (Objective 2)
   A. increased haptoglobin
   B. thrombocytopenia
   C. reticulocytopenia
   D. decreased LD
2. One of the major criteria that distinguishes DIC from other causes of microangiopathic hemolytic anemia is: (Objective 2)
   A. the presence of schistocytes
   B. thrombocytopenia
   C. decreased hemoglobin
   D. an abnormal coagulation test
3. A patient who has anemia with an increased reticulocyte count, increased bilirubin, and many schistocytes on the blood smear could have: (Objective 2)
   A. MAHA
   B. high cholesterol in the blood
   C. spur cell anemia
   D. immune hemolytic anemia
4. Which of the following organisms does *not* cause damage of the erythrocyte because of an intraerythrocytic life cycle? (Objective 4)
   A. *Plasmodium falciparum*
   B. *Babesia* sp.
   C. *Bartonella* sp.
   D. *Clostridium perfringens*

5. A characteristic finding on a blood smear in MAHA is the presence of: (Objective 3)

   A. target cells

   B. spur cells

   C. schistocytes

   D. echinocytes

6. All of the following are characterized as causes of MAHA *except*: (Objective 1)

   A. TTP

   B. DIC

   C. HUS

   D. March hemoglobinuria

7. All of the following are associated with HUS *except*: (Objective 2)

   A. thrombocytosis

   B. nucleated RBCs in peripheral blood

   C. schistocytes

   D. reticulocytosis

8. MAHA is most frequently caused by: (Objective 1)

   A. physical trauma to the cell

   B. immune destruction

   C. antagonists in the blood

   D. plasma lipid abnormalities

9. Intravascular hemolysis in MAHA would be associated with which of the following parameters? (Objective 2)

	Bilirubin	Haptoglobin
a.	decreased	decreased
b.	decreased	increased
c.	increased	decreased
d.	increased	increased

10. MAHA caused by HUS is usually seen in which age group? (Objective 1)

    A. children younger than 5 years of age

    B. females between 20 and 50 years of age

    C. either sex younger than 50 years of age

    D. males older than 16 years of age

Level II

1. A 43-year-old woman presents to her physician with a 3-week history of fatigue, constant headache, and low-grade fever. Selected laboratory results include:

Hb	7.5 g/dL (75 g/L)	Platelet count	16×10^9/L
Hct	0.23 L/L (23%)	Reticulocytes	11%
RDW	15		

   Peripheral blood smear showed schistocytes. Which of the following drugs that the patient was taking could cause these symptoms and lab values? (Objectives 2, 4)

   A. ticlopidine

   B. aspirin

   C. estrogens

   D. quinine

2. A 34-year-old woman is brought into the ER after falling off a ladder while painting her house. Selected lab results include:

Hb	8.0 g/dL (80 g/L)	PT	36 seconds
Hct	0.25 L/L (25%)	APTT	>75 seconds
Platelet count	20×10^9/L	Fibrinogen	100 mg/dL

   Peripheral blood smear shows schistocytes. Given these results, what is the most likely diagnosis? (Objectives 2, 4)

   A. HELLP syndrome

   B. TTP

   C. DIC

   D. traumatic hemolytic anemia

3. Results of tests obtained on a small child who had easy bruising, tiredness, difficulty breathing, and decreased urinary output were hemoglobin: 65 g/L; platelets: 41×10^9/L; PT and APTT within normal reference intervals. His mother indicated he had an episode of bloody diarrhea about 2 weeks earlier but it had not recurred. Based on the clinical and limited laboratory findings, what is the most likely condition? (Objectives 2, 4)

   A. TTP

   B. bartonellosis

   C. *Clostridium* sp. infection

   D. HUS

4. The most likely age group for developing TTP is: (Objective 2)

   A. female children younger than 1 year

   B. women between 20 and 50 years

   C. either gender younger than 5 years

   D. men older than 16 years

5. Which of the following disorders is *not* characterized by the presence of schistocytes? (Objective 3)
   A. march hemoglobinuria
   B. insertion of a prosthetic valve
   C. third-degree burns
   D. malignant hypertension
6. A patient with a deficiency in the VWF protease ADAMTS13 would be at risk to develop which condition? (Objective 2)
   A. HUS
   B. spur cell anemia
   C. TTP
   D. hereditary acanthocytosis
7. The formation of schistocytes in MAHA is primarily due to: (Objective 1)
   A. pitting by splenic macrophages
   B. defective cell membranes
   C. increased membrane phospholipids
   D. shearing of erythrocytes by fibrin threads
8. Plasma exchange is used as a primary treatment for which of the following? (Objective 2)
   A. HUS
   B. TTP
   C. abetalipoproteinemia
   D. DIC
9. The peripheral blood smear from a 6-months pregnant woman showed the presence of schistocytes. Platelet count was $<60 \times 10^9$/L, and her hemoglobin was 75 g/L (7.5 g/dL). She has no history of chronic disease. What laboratory test(s) might give a clue to the underlying cause? (Objective 4)
   A. haptoglobin
   B. reticulocyte count
   C. liver enzymes
   D. APTT and PT
10. Hemolytic toxins are the major cause of intravascular hemolysis in diseases or conditions caused by which of the following organisms? (Objective 5)
    A. *Plasmodium falciparum*
    B. *Babesia* sp.
    C. *Bartonella bacilliformis*
    D. *Clostridium perfringens*

Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

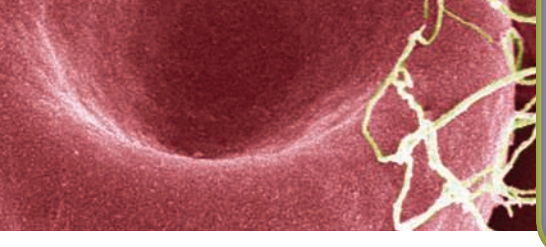
The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Moake JL. Thrombotic microangiopathies. *N Engl J Med*. 2002;347:589–600.
2. Noris M, Ramuzzi G. Hemolytic uremic syndrome. *J Am Soc Nephrol*. 2005;16:1035–50.
3. Banatvala N, Griffin PM, Greene KD et al. The United States national prospective hemolytic uremic syndrome study: microbiologic, serologic, clinical, and epidemiological findings. *J Infect Dis*. 2010;183:1063–70.
4. Pennington H. *Escherichia coli* O157. *Lancet*. 2011;376:1428–35.
5. Ochoa TJ, Cleary TG. Epidemiology and spectrum of disease of *Escherichia coli* O157:H7. *Curr Opin Infect Dis*. 2003;16:259–63.
6. Tarr PI, Gordon CA, Chandler WL. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet*. 2005;365:1073–86.
7. Thorpe CM. Shiga toxin-producing *Escherichia coli* infection. *Clin Infect Dis*. 2004;38:1298–1303.
8. Frank C, Weber D, Cramer JP et al. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 in Germany. *N. Engl J Med*. 2011;365:1771–80.
9. Crump JA, Sulka AC, Langer AH et al. An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. *N Engl J Med*. 2002;347:555–60.
10. Anjay MA, Anoop P, Britland A. Leukocytosis as a predictor of progression to haemolytic uraemic syndrome in *Escherichia coli* O157:H7 infection. *Arch Dis Child*. 2007;92:820–23.
11. Fernandez GC, Gomez SA, Ramos MV et al. The functional state of neutrophils correlates with the severity of renal dysfunction in children with hemolytic uremic syndrome. *Pediatr Res*. 2007;61:123–28.
12. Buteau C, Proulx F, Chaibou M et al. Leukocytosis in children with *Escherichia coli* O157:H7 enteritis developing the hemolytic uremic syndrome. *Pediatr Infect Dis J*. 2000;19:642–47.
13. Wong CS, Jelacic S, Habeeb RL et al. The risk of the hemolytic-uremic syndrome after treatment of *Escherichia coli* O157:H7 infections. *N Engl J Med*. 2000;342:1930–36.
14. Beatty ME, Griffin PM, Tulu AN et al. Culturing practices and antibiotic use in children with diarrhea. *Pediatrics*. 2004;113:628–29.
15. Slutsker L, Ries AA, Maloney K et al. A nationwide-case control study of *Escherichia coli* O157:H7 infection in the United States. *J Infect Dis*. 1998;177:962–66.
16. Siegler RL, Pavla AT, Sherbotie JR. Recurrent hemolytic uremic syndrome. *Clin Pediatr*. 2002;41:705–9.
17. Siegler R, Oakes R. Hemolytic uremic syndrome: pathogenesis, treatment, and outcome. *Curr Opin Pediatr*. 2005;17:200–4.
18. Westra D, Wetzels JFM, Volokhina EB et al. A new era in the diagnosis and treatment of atypical haemolytic uraemic syndrome. *Neth J Med*. 2012;70:121–29.

19. James SH, Meyers AM. Microangiopathic hemolytic anemia as a complication of diabetes mellitus. *Am J Med Sci*. 1998;315:211–15.
20. Gherman RB, Tramont J, Connito DJ. Postpartum hemolytic-uremic syndrome associated with lupus anticoagulant. *J Reprod Med*. 1999;44:471–74.
21. George J. The association of pregnancy with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Curr Opin Hematol*. 2003;10:339–44.
22. Alexandrescu DT, Maddukuri P, Wiernik PH et al. Thrombotic thrombocytopenic purpura/hemolytic uremic syndrome associated with high-dose interleukin-2 for the treatment of metastatic melanoma. *J Immunother*. 2005;28:144–47.
23. Matinez MT, Chucher CH, Stussi G et al. Transplant-associated microangiopathy (TAM) in recipients of allogeneic hematopoietic stem cell transplants. *Bone Marrow Transplant*. 2005;36:993–1000.
24. Turner D, Schreiber R, Grant D et al. Hemolytic uremic syndrome after pediatric liver transplantation. *J Pediatr Gastroenterol Nutr*. 2006;43:109–12.
25. Daly AS, Xenocostas A, Lipton JH. Transplantation-associated thrombotic microangiopathy: 22 years later. *Bone Marrow Transplant*. 2002;30:709–15.
26. Cabrera G, Fortenberry J, Warshaw BL et al. Hemolytic uremic syndrome associated with invasive *Streptococcus pneumoniae* infection. *Pediatrics*. 1998;101:689–703.
27. Cochran JB, Panzarino VM, Maes LY et al. Pneumococcal-induced T antigen activation in hemolytic uremic syndrome and anemia. *Pediatr Nephrol*. 2004;19:317–21.
28. Huang DTN, Chi H, Lee HC et al. T-antigen activation for prediction of pneumococcus-induced hemolytic uremic syndrome and hemolytic anemia. *Ped Infect Dis J*. 2006;25:608–10.
29. Simonelli GD, Dumont-Dos Santos K, Pachlopnik JM et al. Hemolytic uremic syndrome linked to infectious mononucleosis. *Pediatr Nephrol*. 2003;18:1193–94.
30. Banarjee R, Hersh AL, Newland J et al. *Streptococcus pneumoniae*-associated hemolytic uremic syndrome among children in North America. *Pediatr Infect Dis J*. 2011;30:7369.
31. Medina PJ, Sipolis JM, George JN. Drug-associated thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Curr Opin Hematol*. 2001;8:286–93.
32. Zipfel PF, Misselwitz J, Licht C et al. The role of defective complement control in hemolytic uremic syndrome. *Semin Thromb Hemost*. 2006;32:146–54.
33. Zipfel PF, Skerka C. Complement dysfunction in hemolytic uremic syndrome. *Curr Opin Rheumatol*. 2006;18:548–55.
34. Cho HY, Lee BS, Moon KC et al. Complement factor H deficiency-associated atypical hemolytic uremic syndrome in a neonate. *Pediatr Nephrol*. 2007;22:874–80.
35. Landau D, Shalev H, Levy-Finer G et al. Familial hemolytic uremic syndrome associated with complement factor H deficiency. *J Pediatr*. 2001;138:412–17.
36. Noris M, Brioschi S, Caprioli J et al. Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet*. 2003;362:1542–47.
37. Dragon-Durey MA, Loirat C, Cloarec S et al. Anti-factor H autoantibodies associated with atypical hemolytic uremic syndrome. *J Am Soc Nephrol*. 2005;16:555–63.
38. Jozsi M, Licht C, Strobel S et al. Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. *Blood*. 2008;111:1512–14.
39. te Loo DM, Monnens LA, van Der Delden TJ et al. Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood*. 2000;95:3396–402.
40. Kerr H, Richards A. Complement-mediated injury and protection of endothelium: lessons from atypical haemolytic uraemic syndrome. *Immunobiology*. 2012;217:195–203.
41. Johnson S, Waters A. Is complement a culprit in infection-induced forms of haemolytic uraemic syndrome? *Immunobiology*. 2012;217:235–43.
42. Proulx F, Seidman EG, Karpman D. Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. *Pediatr Res*. 2001;50:163–71.
43. Goldwater PN, Bettelheim KA. Treatment of enterohemorrhagic *Escherichia coli* (EHEC) infection and hemolytic uremic syndrome (HUS). *BMC Med*. 2012;10:12.
44. Ostronoff M, Ostronoff F, Calixto R et al. Life-threatening hemolytic-uremic syndrome treated with rituximab in an allogeneic bone marrow transplant recipient. *Bone Marrow Transplant*. 2007;39:649–51.
45. Tazzari PL, Ricci F, Carnicelli D et al. Flow cytometry detection of Shiga toxins in the blood from children with hemolytic uremic syndrome. *Cytometry B Clin Cytom*. 2004;61:40–44.
46. MacConnachie AA, Todd WT. Potential therapeutic agents for the prevention and treatment of haemolytic uraemic syndrome in Shiga toxin producing *Escherichia coli* infection. *Curr Opin Infect Dis*. 2004;17:479–82.
47. Köse O, Zimmerhackl LB, Jungraithmayr T et al. New treatment options for atypical hemolytic uremic syndrome with the complement inhibitor eculizumab. *Semin Thromb Hemost*. 2010;36:669–72.
48. Yarranton H, Machin SJ. An update on the pathogenesis and management of acquired thrombotic thrombocytopenic purpura. *Curr Opin Neurol*. 2003;16:367–73.
49. Allford SL, Machin SJ. Current understanding of the pathophysiology of thrombotic thrombocytopenic purpura. *J Clin Pathol*. 2000;53:497–501.
50. Raife TJ, Montgomery RR. von Willebrand factor and thrombotic thrombocytopenic purpura. *Curr Opin Hematol*. 2000;7:278–83.
51. Zheng X, Majerus EM, Sadler JE. ADAMTS13 and TTP. *Curr Opin Hematol*. 2002;9:389–94.
52. Tsai HM. Deficiency of ADAMTS13 causes thrombotic thrombocytopenic purpura. *Arterioscler Thromb Vasc Biol*. 2003;23:388–96.
53. Veyradier A, Obert B, Houllien A et al. Specific von Willebrand factor—cleaving protease in thrombotic microangiopathies: a study of 111 cases. *Blood*. 2001;98:1765–72.
54. Levy GG, Nichols WC, Lian EC et al. Mutations in a membrane of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. 2001;413:488–94.
55. Burns ER, Lou Y, Pathak A. Morphologic diagnosis of thrombotic thrombocytopenic purpura. *Am J Hematol*. 2004;75:18–21.
56. Park YA, Waldrum MR, Marques MB. Platelet count and prothrombin time help distinguish thrombotic thrombocytopenic purpura-hemolytic uremic syndrome from disseminated intravascular coagulation in adults. *Am J Clin Pathol*. 2010;133:460–65.
57. Muncunill J. Thrombotic thrombocytopenic purpura (TTP): is any plasma useful to treat the TTP? *ISBT Sci Ser*. 2007;2:233–39.
58. Ziman A, Mitri M, Klapper E et al. Combination vincristine and plasma exchange as initial therapy in patients with thrombotic thrombocytopenic purpura: one institution's experience and review of literature. *Transfusion*. 2005;45:41–49.
59. Shariatmader S, Nassiri M, Vincek V. Effect of plasma exchange on cytokines measured by multianalyte bead array in thrombotic thrombocytopenic purpura. *Am J Hematol*. 2005;79:83–88.
60. von Baeyer H. Plasmapheresis in thrombotic microangiopathy-associated syndromes: review of outcome data derived from clinical trials and open studies. *Ther Apher*. 2002;6:320–28.
61. Clark WF. Thrombotic microangiopathy: current knowledge and outcomes with plasma exchange. *Semin Dial*. 2012;25:214–19.
62. George JN. How I treat patients with thrombotic thrombocytopenic purpura. *Blood*. 2010;116:4060–69.
63. George JN. Corticosteroids and rituximab as adjunctive treatments for thrombotic thrombocytopenic purpura. *Am J Hematol*. 2012;87:S88–91.
64. Fakhouri F, Vernant JP, Veyradier A et al. Efficiency of curative and prophylactic treatment with rituximab in ADAMTS13-deficient thrombotic thrombocytopenic purpura. *Blood*. 2005;106:1932–37.
65. Levi M, Ten Cate H. Current concepts: disseminated intravascular coagulation. *N Engl J Med*. 1999;341:586–92.
66. Yu M, Nardella A, Pechet L. Screening tests of disseminated intravascular coagulation: guidelines for rapid and specific laboratory diagnosis. *Crit Care Med*. 2000;28:1777–80.
67. Hess JR, Lawson JH. The coagulopathy of trauma versus disseminated intravascular coagulation. *J Trauma*. 2006;60:512–19.
68. Favaloro EJ. Laboratory testing in disseminated intravascular coagulation. *Semin Thromb Hemost*. 2010;36:458–67.
69. Karam K, Svendsen E, Abildgaard U. The HELLP syndrome: clinical issues and management. A review. *BMC Pregnancy Childbirth*. 2009;9:8.
70. Levi M, Seligsohn U. Disseminated intravascular coagulation. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010. Chapter 130. www.accessmedicine.com/content.aspx?aID=6235198; accessed April 3, 2014.
71. Lee RM, Brown MA, Branch W et al. Anticardiolipin and anti-B_2-glycoprotein-I antibodies in preeclampsia. *Obstet Gynecol*. 2003;102:294–300.
72. Appenzeller S, Souza FHC, de Souza AWS et al. HELLP syndrome and its relationship with antiphospholipid syndrome and antiphospohlipid antibodies. *Semin Arthritis Rheum*. 2011;41:517–23.
73. O'Brien JM, Barton JR. Controversies with the diagnosis and management of HELLP syndrome. *Clin Obstet Gynecol*. 2005;48:460–77.
74. Cavtaykar S, Ugurlu EN, Karaer A et al. Are clinical symptoms more predictive than laboratory parameters for adverse maternal outcome in HELLP syndrome? *Acta Obstet Gynecol Scand*. 2007;86:648–51.

75. Vigil-De Gracia P. Addition of platelet transfusions to corticosteroids does not increase the recovery of severe HELLP syndrome. *Eur J Obstet Gynecol Reprod Biol*. 2006;128:194–98.
76. van Runnard Heimel PJ, Franx A, Schobben AF et al. Corticosteroids, pregnancy, and HELLP syndrome: a review. *Obstet Gynecol Surv*. 2005;60:57–70.
77. van den Born BJH, Honnebier UPF, Koopmans RP et al. Microangiopathic hemolysis and renal failure in malignant hypertension. *Hypertension*. 2005;45:246–51.
78. van den Born BJH, van der Hoeven NV, Groot E et al. Association between thrombotic microangiopathy and reduced ADAMTS13 activity in malignant hypertension. *Hypertension*. 2008;51:862–66.
79. Choi CM, Schmaier AH, Snell MR et al. Thrombotic microangiopathy in haematopoietic stem cell transplantation. *Drugs*. 2009;69:183–98.
80. Mecozzi G, Milano ADF, DeCarlo M et al. Intravascular hemolysis in patients with new-generation prosthetic heart valves: a prospective study. *J Thorac Cardiovasc Surg*. 2002;123:550–56.
81. Najib MQ, Vinales KL, Paripati HR et al. An unusual presentation of hemolytic anemia in a patient with prosthetic mitral valve. *Echocardiography*. 2011;28:E112–14.
82. Pratt VC, Tredger EE, Clandnin MT et al. Fatty acid content of plasma lipids and erythrocyte phospholipids are altered following burn injury. *Lipids*. 2001;36:675–82.
83. Telford RD, Sly GJ, Hahn AG et al. Footstrike is the major cause of hemolysis during running. *J Appl Physiol*. 2003;94:38–42.
84. Tobal D, Olascoaga A, Moreira G et al. Rust urine after intense hand drumming is caused by extracorpuscular hemolysis. *Clin J Am Soc Nephrol*. 2008;3:1022–27.
85. Senturk UK, Gunduz F, Kuru O et al. Exercise-induced oxidative stress leads hemolysis in sedentary but not trained humans. *J Appl Physiol*. 2005;99:1434–41.
86. Robinson Y, Cristancho E, Boning D. Intravascular hemolysis and mean red blood cell age in athletes. *Med Sci Sports Exerc*. 2005;38:480–83.
87. Yusof A, Leithauser RM, Roth HJ et al. Exercise-induced hemolysis is caused by protein modification and most evident during the early phase of an ultraendurance race. *Appl Physiol*. 2007;102:582–86.
88. Casals-Pascual C, Roberts DJ. Severe malarial anaemia. *Curr Mol Med*. 2006;6:155–68.
89. Powell VI, Grima K. Exchange transfusion for malaria and *Babesia* infection. *Transfus Med Rev*. 2002;16:239–50.
90. Waitumbi JN, Opollo MO, Muga RO et al. Red cell surface changes and erythrophagocytosis in children with severe *Plasmodium falciparum* anemia. *Blood*. 2000;95:1481–86.
91. Weinberg GA. Laboratory diagnosis of ehrlichiosis and babesiosis. *Pediatr Infect Dis J*. 2001;20:435–38.
92. Krause PJ. Babesiosis diagnosis and treatment. *Vector Borne Zoonootic Dis*. 2003;3:45–51.
93. Dehio C. Molecular and cellular basis of *Bartonella* pathogenesis. *Ann Rev Microbiol*. 2004;58:365–90.
94. Huarcaya E, Best I, Rodriguez-Tafur J et al. Cytokines and T-lymphocyte count in patients in the acute and chronic phases of *Bartonella bacilliformis* infection in an endemic area in Peru: a pilot study. *Rev Inst Med Trop Sao Paulo*. 2011;53:149–54.
95. Hendrix LR. Contact-dependent hemolytic activity distinct from deforming activity of *Bartonella bacilliformis*. *FEMS Microbiol Lett*. 2000;182:119–24.
96. Watt J, Amini A, Mosier J et al. Treatment of severe hemolytic anemia caused by *Clostridium perfringens* sepsis in a liver transplant recipient. *Surg Infect*. 2012;13:60–62.
97. Hogan CJ, Barbero KC, Winkel K. Loxoscelism: old obstacles, new directions. *Ann Emerg Med*. 2004;44:608–24.
98. Hostetler MA, Dribben W, Wilson DB et al. Sudden unexplained hemolysis occurring in an infant due to presumed Loxosceles envenomation. *J Emerg Med*. 2003;25:277–82.
99. Hubbard JJ, James LP. Complications and outcomes of brown recluse spider bites in children. *Clin Pediatr*. 2011;50:252–58.
100. McDade J, Aygun B, Ware RE. Brown recluse spider (*Loxosceles reclusa*) envenomation leading to acute hemolytic anemia in six adolescents. *J Pediatr*. 2010;156:155–57.
101. Tambourgi DV, Morgan BP, de Andrade RM et al. *Loxosceles intermedia* spider envenomation induces activation of an endogenous metalloproteinase, resulting in cleavage of glycophorins from the erythrocyte surface and facilitating complement-mediated lysis. *Blood*. 2000;95:683–91.
102. Isbister GK. Snakebite doesn't cause disseminated intravascular coagulation; coagulopathy and thrombotic microangiopathy in snake envenomation. *Semin Thromb Hemost*. 2010;36:444–51.



21 Nonmalignant Disorders of Leukocytes: Granulocytes and Monocytes

KRISTIN LANDIS-PIWOWAR, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Recognize neutrophilia from hematologic data and name the common disorders associated with neutrophilia.
2. Explain the quantitative and qualitative neutrophil response to acute bacterial infections.
3. Identify immature granulocytes and morphologic changes (toxic granulation, Döhle bodies, intracellular organisms, and vacuoles) often seen in reactive neutrophilia.
4. Define and recognize *leukemoid reaction*, *leukoerythroblastosis*, and *pyknotic nuclei* on stained blood films and microscopic pictures.
5. Distinguish leukemoid reaction from chronic myeloid leukemia based on laboratory data including the leukocyte alkaline phosphatase stain.
6. Identify neutropenia from hematologic data and list the common disorders associated with neutropenia.
7. Recognize the conditions associated with false or pseudo-neutropenia.
8. Identify neutrophil nuclear alterations including Pelger-Huët, hypersegmentation, and pyknotic forms on stained blood films and microscopic pictures.
9. Recognize morulae, Alder-Reilly granules, or Chédiak-Higashi inclusions on stained blood films and microscopic pictures.
10. State the common conditions associated with abnormal eosinophil, basophil, and monocyte counts.
11. Define *Gaucher* and *Niemann-Pick diseases.*

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Assess the etiology, associated conditions, and peripheral blood findings for immediate, acute, chronic, and reactive neutrophilia.
2. Compare and contrast the hematologic and clinical features for leukemoid reaction and chronic myeloid leukemia (CML).
3. Organize neutropenia to include etiology and associated conditions as well as blood and bone marrow findings.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

4. Recognize, evaluate, and select appropriate corrective action for false neutropenia.
5. Appraise the nuclear abnormalities of neutrophils including Pelger-Huët, pseudo-Pelger-Huët, hypersegmentation, and pyknotic nuclei, and reconcile them with the appropriate clinical conditions of the patient.
6. Appraise the cytoplasmic abnormalities of neutrophils including toxic granulation, Döhle bodies, vacuoles, intracellular organisms, and morulae and reconcile them with the patient's appropriate clinical condition.
7. Recognize and summarize the clinical features of the inherited granulocyte functional abnormalities (Chédiak-Higashi, Alder-Reilly, May-Hegglin, and chronic granulomatous diseases) and differentiate their cellular abnormalities.
8. Evaluate alterations in the relative and/or absolute numbers of eosinophils, basophils, and monocytes and associate them with the clinical condition of the patient.
9. Evaluate the etiology, laboratory findings, and clinical features of the lysosomal storage disorders.
10. Identify and differentiate the abnormal macrophages seen in Gaucher disease, Niemann-Pick disease, and sea-blue histiocytosis syndrome.
11. Construct an efficient and cost-effective reflex testing pathway for follow-up neutrophilia, neutropenia, and qualitative granulocyte abnormalities.
12. Evaluate a case study from a patient with a nonmalignant granulocyte disorder.

Key Terms

Agranulocytosis
Basophilia
Demargination
Döhle body
Egress
Eosinophilia
Hypereosinophilic syndrome (HES)
Leukemoid reaction
Leukocytosis
Leukoerythroblastic reaction
Leukopenia
Lysosomal storage disorders
Mastocytosis
Monocytopenia
Monocytosis
Morulae
Myelophthisis
Neutropenia
Neutrophilia
Pelger-Huët anomaly (PHA)
Pseudo-neutrophilia
Reactive neutrophilia
Sea-blue histiocytosis syndrome
Shift to the left/left shift
Toxic granules

Background Basics

The information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review before starting this unit of study:

Level I

- Summarize the production, kinetics, distribution, life span, and basic function of neutrophils and monocytes. (Chapter 7)
- Describe how leukocytes are counted and differentiated; recognize normal and immature granulocytes. (Chapters 7, 10, 37)

Level II

- Describe the role of specific neutrophil granules and enzymes in antimicrobial systems. (Chapter 7)
- Identify normal macrophages and discuss their role in the bone marrow and the rest of the monocyte-macrophage system. (Chapter 7)
- Describe leukocyte maturation and proliferation pools in the bone marrow; describe the role of cytokines in bone marrow release of leukocytes; describe the process of leukocyte egress to tissue. (Chapters 3, 4, 7)
- Correlate the function of the hematopoietic organs to leukocyte distribution and demise. (Chapter 3)

CASE STUDY

We will address this case throughout the chapter.

Dennis, a 24-year-old man, was taken to emergency surgery to repair several bone fractures sustained in an automobile accident. His previous medical history was unremarkable. He was in excellent health prior to the accident.

Consider why Dennis' condition could result in abnormal hematologic test results and the possible complications that could occur during Dennis' treatment and recovery.

OVERVIEW

This chapter discusses benign changes in granulocytes and monocytes as a response to various nonmalignant disease states and toxic challenges. These changes include both quantitative and qualitative variations that can be detected by laboratory professionals. The chapter is organized by class of leukocyte (neutrophils, eosinophils, basophils, and monocytes) and by the changes in quantity and quality (morphology) of those leukocytes. These variations in leukocyte number and appearance are correlated with acquired and inherited physiologic states. The laboratory professional must recognize these abnormalities and correlate them with the patient's clinical condition.

INTRODUCTION

Changes in leukocyte concentration and morphology are often the body's normal responses to various disease processes and toxic challenges. Most often, one class of leukocyte is affected more than the others, providing an important clue to diagnosis. The type of cell affected depends in a large part on its function (e.g., bacterial infection commonly results in an absolute neutrophilia, viral infections are characterized by an absolute lymphocytosis, and certain parasitic infections cause an eosinophilia). Thus, the absolute concentrations of each leukocyte class aids in the differential diagnosis of a patient, especially when the total leukocyte concentration is abnormal.

Leukocytosis refers to a condition in which the total leukocyte count is more than 11.0 $\times$ 10^9/L in an adult. Refer to Table B on the inside cover for leukocyte and differential reference intervals. Although leukocytosis usually occurs because of an increase in neutrophils, it can also be related to an increase in lymphocytes or (rarely) in monocytes, eosinophils, or basophils.[1] Quantitative variations of leukocytes are evaluated by performing a total leukocyte count and a differential count. The absolute concentration of each type of leukocyte can be calculated from these two values as follows (Chapter 7):

Differential count (in decimal form) $\times$ WBC count ($\times 10^9$/L) = Absolute cell count ($\times 10^9$/L)

☑ CHECKPOINT 21-1

An adult patient's total leukocyte count is 5.0 $\times$ 10^9/L. There are 60% segmented neutrophils, 35% lymphocytes, and 5% monocytes on the differential. Calculate the absolute number of each cell type. Is each of these relative and absolute cell counts normal or abnormal?

Leukopenia refers to a condition in which the total leukocyte count falls $<$4.5 $\times$ 10^9/L. This condition is usually the result of a decrease in neutrophils, but lymphocytes and other leukocytes can also contribute.

Morphologic or qualitative variations in leukocytes are noted by examination of the stained blood smear (Chapter 10). Some qualitative changes affect cell function, whereas others do not. Variations in the appearance of the cell together with its concentration can provide specific clues to the pathologic process.

NEUTROPHIL DISORDERS

Because neutrophils are the most numerous type of leukocyte that circulates in the peripheral blood, quantitative disorders of neutrophils are often accompanied by changes in the total leukocyte count. Although neutrophilia (increase in neutrophils) is more common than neutropenia (decrease in neutrophils), the consequences to the health of a patient are more severe when the neutrophil count is low. Automated hematology analyzers detect neutrophil counts outside the reference interval, but they do not detect qualitative changes in the neutrophils. Detection of these changes requires careful microscopic examination of stained blood films and provides information for differential diagnosis, especially in patients with recurring bacterial infections.

Quantitative Disorders

Quantitative neutrophil abnormalities can result from malignant or benign disorders. Malignant disorders are caused by neoplastic transformation of hematopoietic stem cells and are discussed in Chapters 23–28. Benign disorders are usually acquired and most often occur as a consequence of a physiologic insult (stressful stimulus that affects normal function and can result in morbidity). Table 21-1 ★ lists three interrelated mechanisms affecting neutrophil concentration in the peripheral blood.

Neutrophilia

The reference interval for neutrophil concentration varies with age and race, which emphasizes the importance of evaluating the count for each demographic group. **Neutrophilia** refers to an increase in the total circulating absolute neutrophil concentration (ANC). In adults, neutrophilia occurs when the ANC exceeds 7.0 $\times$ 10^9/L. (See Table B on the inside cover for age- and race-specific reference intervals.)

Neutrophilia that is not caused by malignancy (benign) most often occurs as a response to a physiologic or pathologic process and is termed **reactive neutrophilia**; it can be immediate, acute, or chronic and can involve any or all of the three mechanisms listed in Table 21-1.

Immediate Neutrophilia

Of the neutrophils inside a blood vessel, approximately 50% freely circulate, and the remaining 50% are loosely attached to the endothelial cells of the blood vessel (marginated) (Chapter 7). Immediate neutrophilia can occur without pathologic stimulus and is probably a simple redistribution of the marginating pool to the circulating pool. Routine laboratory analysis of a peripheral blood sample detects only those neutrophils in the circulating pool.

Although the increase in circulating neutrophils occurs rapidly in immediate neutrophilia, it is transient (lasting only 20–30 minutes). Furthermore, immediate neutrophila is independent of bone marrow output and tissue **egress** (movement of neutrophils out of the circulation and into the tissues). Therefore, immediate neutrophilia is also referred to as **pseudo-neutrophilia** or **demargination** because no actual change in the number of neutrophils within the vasculature occurs. The increased circulating neutrophils are typically mature, normal cells. This redistribution of neutrophils causes the physiologic neutrophilia that accompanies active exercise, epinephrine administration, anesthesia, and anxiety and can increase the neutrophil count by as much as two-fold.[2]

Acute Neutrophilia

Acute neutrophilia occurs when neutrophils egress from the bone marrow storage pool into the peripheral blood. Within hours following a pathologic stimulus (e.g., bacterial infection, toxin) the circulating pool of neutrophils can increase by as much as 10-fold.[3]

★ **TABLE 21-1** Factors Affecting Neutrophil Concentration in Peripheral Blood

- Bone marrow production and release of neutrophils
- Rate of neutrophil egress to tissue or survival time in blood
- Ratio of marginating to circulating neutrophils in peripheral blood

The neutrophilia is far more pronounced than in pseudoneutrophilia, and the proportion of immature neutrophils can be increased. More bands appear if the tissue demand for neutrophils creates an acute shortage of segmented neutrophils in the bone marrow storage pool. Continued demand in extreme circumstances can result in the release of metamyelocytes.[2] As bone marrow production increases and the storage pool is replenished, the leukocyte differential returns to normal.

Chronic Neutrophilia

Chronic neutrophilia generally follows acute neutrophilia and occurs if the stimulus for neutrophils continues beyond a few days. This results in a depleted storage pool and increased production of the mitotic pool in an attempt to meet the demand for neutrophils. In this state, the marrow shows increased numbers of early neutrophil precursors including myeloblasts, promyelocytes, and myelocytes. The blood contains increased numbers of bands, metamyelocytes, myelocytes, and (rarely) promyelocytes. An increase in the concentration of immature forms of leukocytes in the circulation is termed a **shift to the left** or **left shift**.

Conditions Associated with Neutrophilia

Chronic neutrophilia caused by benign or toxic conditions is usually characterized by a total leukocyte count of less than 50×10^9/L and a left shift. If immature cells are present they are usually bands and metamyelocytes. See Table 21-2 ★ for conditions associated with neutrophilia. In some instances, the neutrophil count can be normal, but toxic morphological changes can be observed including toxic granulation, Döhle bodies, and cytoplasmic vacuoles (discussed later in this chapter and illustrated in Figure 21-1 ■). In addition, the leukocyte alkaline phosphatase score can be elevated (Chapters 23, 37).

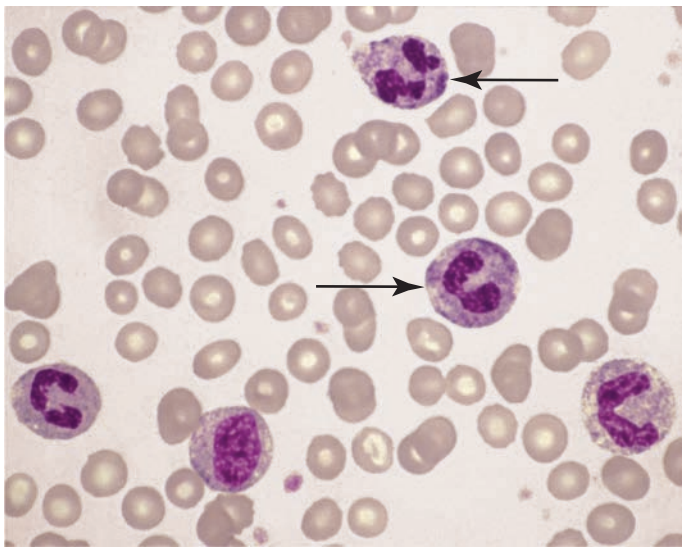
★ **TABLE 21-2** Conditions Associated with Neutrophilia

- Acute bacterial and fungal infections
- Inflammatory processes
  - Tissue damage from burns, trauma, or surgery
  - Collagen, vascular, and autoimmune disorders
  - Hypersensitivity reactions
- Metabolic alterations
  - Uremia
  - Eclampsia
  - Gout
- Nonmyeloid neoplasms
- Acute hemorrhage or hemolysis
- Rebound from bone marrow transplant or treatment with colony -stimulating growth factors
- Certain chemicals, toxins, and drugs
- Physiologic neutrophilia
  - Strenuous exercise
  - Stress
  - Pain
  - Temperature extremes
  - Childbirth
  - Newborns
- Chronic myeloproliferative disorders[a]

[a] In these disorders, increases in neutrophils are from a neoplasm of the hematopoietic stem cell.

Bacterial Infection Bacterial infection is the most common cause of neutrophilia, especially infection caused by pyogenic organisms such as staphylococci and streptococci. Depending on the virulence of the microorganism, extent of infection, and response of the host, the neutrophil count can range from $7.0–70 \times 10^9$/L, although the count is usually in the range of $10–25 \times 10^9$/L. As the demand for neutrophils at the site of infection increases, the early response of the bone marrow is to increase output of storage neutrophils to the peripheral blood, causing a left shift. The inflow of neutrophils from the bone marrow to the blood continues until it exceeds the neutrophil outflow to the tissues, causing an absolute neutrophilia. In very severe infections, the storage pool of neutrophils can become exhausted, the mitotic pool can be unable to keep up with the demand, and a neutropenia develops.[4] Neutropenia in overwhelming infection is a poor prognostic indicator. Chronic bacterial infection can lead to chronic stimulation of the marrow whereby the production of neutrophils remains high and a new steady state of production develops.

Neutrophilia is neither a unique nor an absolute finding in bacterial infections. Infections with other organisms such as fungi, rickettsia, spirochetes, and parasites also can cause a neutrophilia. Certain bacterial infections are characterized by neutropenia rather than neutrophilia.[4] Bacterial infections rarely lead to lymphocytosis rather than neutrophilia, as is the case with whooping cough caused by *Bordetella pertussis* infection. Although typically associated with a lymphocytosis, viruses can also cause neutrophilia in the earliest stages of infection.



■ **FIGURE 21-1** A leukemoid reaction. There is an increased leukocyte count and a left shift. The cells have heavy toxic granulation and Döhle bodies (arrows), suggesting an infectious or toxic reactive leukocytosis (peripheral blood, Wright-Giemsa stain, 1000× magnification).

CASE STUDY (continued from page 389)

Laboratory results on Dennis, the trauma patient, two days after surgery are as follows:

		WBC Differential	
WBC	14.5 × 10^9/L	Segmented neutrophils	5%
Hb	12.9 g/dL	Band neutrophils	50%
PLT	180 × 10^9/L	Lymphocytes	40%
		Monocytes	5%

Urine, blood, and wound cultures were ordered.

1. What results, if any, are abnormal?
2. What is the most likely reason for these results?

Tissue Destruction/Injury, Inflammation, and Metabolic Alterations Conditions other than infection that can result in a neutrophilia include tissue necrosis, inflammation, certain metabolic conditions, and drug intoxication. All of these conditions produce neutrophilia by increasing neutrophil egress from the bone marrow into the circulation in response to increased neutrophil diapedesis to the tissue. Examples of these conditions include rheumatoid arthritis, tissue infarctions, burns, neoplasms, trauma, uremia, and gout.

Although leukocytes defend the body against foreign substances, they also contribute to the inflammatory process. Damaged tissue releases cytokines that act as chemotaxins, causing neutrophils to leave the vessels and move toward the injury site. In gout, for example, deposits of uric acid crystals in joints attract neutrophils to the area. In the process of phagocytosis and death, the leukocytes release toxic intracellular enzymes (granules) and oxygen metabolites. These toxic substances mediate the inflammatory process by injuring other body cells and propagating the formation of additional chemotactic factors that attract more leukocytes.

Leukemoid Reaction Extreme neutrophilic reactions to severe infections or necrotizing tissue can produce a **leukemoid reaction** (Figure 21-1). A leukemoid reaction is a benign proliferation of leukocytes characterized by (1) a total leukocyte count usually <50 × 10^9/L and (2) numerous circulating leukocyte precursors.[5] In a neutrophilic-leukemoid reaction, the number of circulating neutrophil precursors is increased including bands, metamyelocytes, myelocytes and promyelocytes, and rarely, blasts.

Leukemoid reactions can produce a blood picture indistinguishable from that of chronic myeloid leukemia (CML) (Chapter 24). If a differential diagnosis cannot be determined by routine hematologic parameters, then genetic studies, molecular analysis, and leukocyte alkaline phosphatase (LAP) stain scores can be helpful (Table 21-3 ★). Contrary to leukemia, a leukemoid reaction is transient, disappearing when the inciting stimulus is removed. A leukemoid reaction can occur in chronic infections (such as tuberculosis) and may present with toxic granules, Döhle bodies, and vacuoles.[5] In addition, leukemoid reactions can accompany carcinoma of the lung, stomach, breast, or liver and other inflammatory processes.[6]

★ **TABLE 21-3** Comparison of Laboratory Results in Leukemoid Reactions and Chronic Myeloid Leukemia (CML)

	Leukemoid Reaction	CML
Leukocyte count	<50 × 10^9/L	Usually >50 × 10^9/L
Leukocyte differential	Shift to the left with bands, metamyelocytes, and myelocytes; neutrophil toxic changes	Shift to the left with immature cells including promyelocytes and blasts; increased eosinophils and basophils
Erythrocyte count	Normal	Often decreased
Platelets	Usually normal	Increased or decreased
LAP	Increased	Decreased
Philadelphia chromosome	Absent	Usually present
BCR/ABL1 gene mutation	Absent	Present
Clinical	Related to primary condition	Systemic (splenomegaly, enlarged nodes, bone pain)

LAP = leukocyte alkaline phosphatase

☑ CHECKPOINT 21-2

How can CML be distinguished from a leukemoid reaction?

Leukoerythroblastic Reaction A **leukoerythroblastic reaction** (Figure 21-2 ■) is characterized by the presence of nucleated erythrocytes and a neutrophilic left shift in the peripheral blood. The total neutrophil count can be increased, decreased, or normal. Erythrocytes in this condition often exhibit poikilocytosis with teardrop shapes and anisocytosis. Leukoerythroblastosis is most often associated with chronic neoplastic myeloproliferative conditions, especially myelofibrosis, **myelophthisis** (replacement of normal hematopoietic tissue in the bone marrow by fibrosis, leukemia, or metastatic cancer cells), and severe hemolytic anemias such as Rh hemolytic disease of the fetus and newborn (HDFN) (Chapter 19).

Stimulated Bone Marrow States Patients whose bone marrow has been stimulated by hematopoietic growth factors or cytokines such as granulocyte monocyte-colony–stimulating factor (GM-CSF) can produce a rapid increase of total white cell counts and release of leukocyte precursors including blasts. Cytokines are used to replenish leukocytes after bone marrow transplant, high-dose chemotherapy, bone marrow failure, or prior to autologous blood donation or stem cell apheresis[7] (Chapter 29).

Corticosteroid therapy produces a neutrophilia that occurs as a result of increased bone marrow output accompanied by decreased migration of neutrophils to the tissues (by inhibition of the ability of neutrophils to adhere to vessel walls).[8] This inhibition of neutrophil diapedesis can in part explain the increased incidence of bacterial

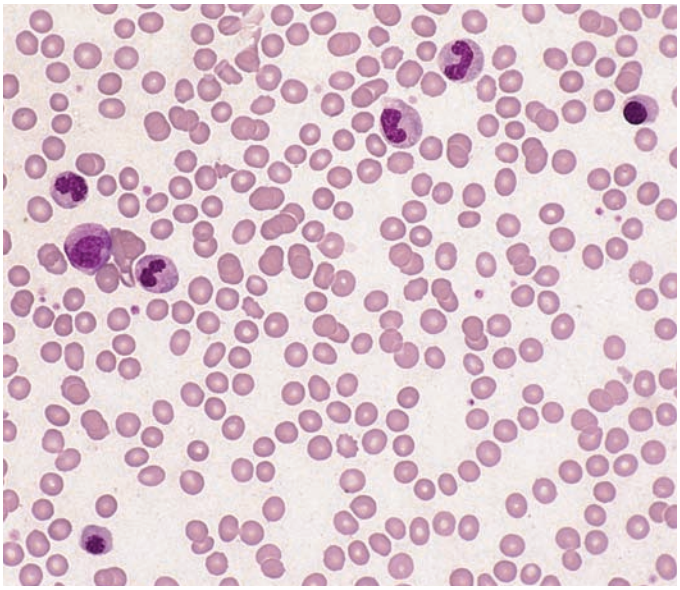


FIGURE 21-2 A leukoerythroblastic reaction. There are nucleated erythrocytes and a left shift with band neutrophils and a myelocyte (peripheral blood, Wright-Giemsa stain, 1000× magnification).

infections in patients on steroid therapy even though the blood neutrophil count is increased. Steroids also decrease the number and inhibit the function of monocytes/macrophages.[9]

Physiologic Leukocytosis Physiologic leukocytosis and neutrophilia are present at birth and for the first few days of life (Table B inside cover). The leukocytosis can be accompanied by a slight left shift. Physiologic stressors including exposure to extreme temperatures, emotional stimuli, exercise, and labor during childbirth can cause neutrophilia, generally without a left shift.

☑ CHECKPOINT 21-3

What is the difference between a leukemoid reaction and a leukoerythroblastic reaction?

Neutropenia

Neutropenia occurs when the neutrophil count is $<1.5–2.0 \times 10^9$/L (varies depending on ethnicity). **Agranulocytosis**, a term that refers to a neutrophil count $<0.5 \times 10^9$/L, is associated with a high probability of infection. Basophils and eosinophils are also commonly depleted in severe neutropenia.

True neutropenia can occur because of (1) decreased bone marrow production, (2) increased cell loss (from immune destruction or increased neutrophil egress to the tissue), or (3) pseudo-neutropenia (increased neutrophilic margination). An artificial neutropenia can result from neutrophil agglutination, disintegration, and laboratory instrument problems. (See Table 21-4 ★ for the most common causes.)

★ **TABLE 21-4** Causes of Leukopenia and/or Neutropenia

Causes	Examples
Infections	Viral and overwhelming bacterial such as sepsis
Physical agents and chemicals	Radiation, benzene
Drugs	Chemotherapy, certain drugs in the classes of sedatives, anti-inflammatory, antibacterial, antithyroid, and antihistamines[a]
Hematologic disorders	Acute leukemia, megaloblastic and aplastic anemia, splenomegaly
Alloantibodies or autoantibodies	Systemic lupus erythematosus
Hereditary or congenital disorders	Familial neutropenia, cyclic neutropenia

[a]For a more complete list of drug-induced neutropenia, see Dale DC. Neutropenia and neutrophilia. In: Kaushansky K, Lichtman MA, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:939–51.

Decreased Bone Marrow Production

Neutropenia can develop as a result of decreased bone marrow production. In this case, the bone marrow shows myeloid hypoplasia, and the myeloid-to-erythroid (M:E) ratio is decreased (Chapter 38). Defective neutrophil production depletes the bone marrow storage pool, decreases neutrophil egress to tissues, and reduces both the peripheral blood circulating and marginating pools. Immature cells may enter the blood in an attempt to alleviate the neutrophil shortage; however, cells younger than bands are less efficient in phagocytosis. The end result is a lack of an adequate number of neutrophils at inflammatory sites, resulting in increased risk for overwhelming infections.

Stem Cell Disorders. Decreased bone marrow production can occur when hematopoietic stem cells fail to proliferate as in aplastic anemia, following radiotherapy or chemotherapy, or with infiltration of hematopoietic tissue by malignant cells (myelophthisis). Of new leukemia cases, 50% present with a total white count that is $<5.0 \times 10^9$/L and an absolute neutrophil count that is $<1.0 \times 10^9$/L.[10] This leukopenia and neutropenia occurs when normal precursor cells in the bone marrow are replaced by malignant cells, but the malignant cells have not yet egressed to the peripheral blood in significant numbers.

Megaloblastic Anemia. Neutropenia is a characteristic finding in megaloblastic anemia (Chapter 16) and myelodysplastic syndromes (Chapter 25). In these cases, however, the marrow is usually hyperplastic. Neutropenia results not from marrow failure but from destruction of abnormal myeloid precursors (ineffective granulopoiesis).

Chemicals/Drugs. A wide variety of drugs and chemicals are associated with leukopenia and neutropenia if given in sufficient dosage (Table 21-4). Chemotherapy and radiation treatments for cancer are nonselective and are common causes of not only neutropenia but also pancytopenia. The decreased bone marrow production of granulocytes predisposes patients receiving treatment for malignancies to frequent and serious infections. Chemotherapeutic drugs

induce apoptosis in mitotically active cells by a variety of mechanisms including direct DNA damage, altering folate receptors, or inhibiting enzymes needed for mitosis. Although the action of chemotherapeutic drugs reduces cancer cell proliferation, hematopoietic precursor cells in the bone marrow also are actively dividing and their proliferation is inhibited as well. Prophylactic use of antibiotics and GM-CSF has reduced mortality in patients receiving chemotherapy, but infections due to neutropenia remain a serious complication.[11] Drug-induced neutropenia also can result from allergic (immunologic) reactions to drugs. Women, older patients, and patients with a history of allergies are more commonly affected.

Congenital Neutropenia. Several rare inherited disorders cause neutropenia related to decreased bone marrow production. Periodic or cyclic neutropenia is a curious form of neutropenia that begins in infancy or childhood and occurs in regular 21- to 30-day cycles. Cyclic neutropenia is inherited as an autosomal dominant trait and is due to mutations in the gene for neutrophil elastase.[12] The severely neutropenic period ($<0.5 \times 10^9$/L) lasts for several days and is marked by frequent infections. Between the neutropenic attacks, the patient is asymptomatic. Severe congenital neutropenia (Kostmann's syndrome) is a rare, often fatal disorder marked by extreme neutropenia ($<0.2 \times 10^9$/L). The total leukocyte count is often in the normal range. It is inherited most often in an autosomal dominant manner and involves mutations in the neutrophil elastase gene (and other genes).[12] Both disorders display neutrophil precursor responsiveness to large doses of G-CSF.[13] Familial neutropenia is a rare benign anomaly characterized by an absolute decrease in neutrophils but usually a normal total leukocyte count. It is transmitted as an autosomal dominant trait and is usually detected by chance. (See Table 21-5 ★ for a more complete list of congenital neutropenic disorders.[2,14])

Increased Cell Loss

Neutropenia can occur as the result of increased neutrophil diapedesis. In severe or early infection, the bone marrow may not produce cells as rapidly as they are being utilized, resulting in neutropenia. Various viral, bacterial, rickettsial, and protozoan infections induce tissue damage that increases the demand for and destruction of neutrophils. Marked toxic changes to the granulocytes often accompany neutropenia resulting from severe infections. Prognosis in these cases is very poor because the infecting organisms are able to prevail over the body's immune system.

Immune Neutropenia. Antibodies directed against neutrophil-specific antigens can cause a decrease in the number of neutrophils. Leukocytes are destroyed in a manner similar to erythrocytes in immune hemolytic anemia (Chapter 17). In some cases, drugs precipitate an immunologic response leading to a sudden disappearance of neutrophils from the circulation. The immunologic mechanism can include direct cell lysis or sensitization and subsequent sequestration of neutrophils in the spleen.[2] The two types of immune neutropenia are alloimmune and autoimmune.

Alloimmune neonatal neutropenia occurs when maternal antibodies are directed against paternal-origin antigens on fetal neutrophils and are transferred across the placenta. Affected infants are susceptible to infections for up to 4 months or until the neutropenia is resolved.[2] This immune process is similar to that found in Rh HDFN except that the firstborn child can be affected. Alloimmune neutropenia can also be the result of a transfusion reaction.

Autoimmune neutropenia (AIN) is categorized as either primary or secondary. Primary AIN is not associated with other diseases and occurs predominantly in young children. This condition of unknown etiology develops as antibody-coated neutrophils are sequestered and destroyed by the spleen. Patients develop fever and recurrent infections. Spontaneous remission usually occurs after a period of 13–20 months. Secondary autoimmune neutropenia generally occurs in older patients, many of whom have been diagnosed with another autoimmune disorder such as systemic lupus erythematosus (SLE) or rheumatoid arthritis. In secondary AIN, antineutrophil antibodies are not the only cause of the neutropenia, and the actual target of the antibodies is not known.[15]

Infections associated with AIN are not usually life threatening and are treated with routine antibiotic therapy. Intravenous doses of immunoglobulin can be used in severe cases. The total leukocyte count is usually normal or near normal, but the neutrophil count is decreased. G-CSF therapy can be indicated for patients with severe infections.

Immune neutropenia can be confirmed by testing for antineutrophil antibodies or neutrophil surface antigens by various methods including agglutination tests, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA). The availability of these complex tests varies widely among laboratories.[16]

Hypersplenism. Hypersplenism can result in a selective splenic culling of neutrophils producing mild neutropenia. The bone marrow in

★ **TABLE 21-5** Congenital Neutropenic Disorders

Disorder	Inheritance
Disorders of production	
Cyclic neutropenia	AD
Familial neutropenia	AD
Fanconi pancytopenia	AR
Reticular dysgenesis	AR
Severe congenital neutrophilia	AD, AR
Wiskott-Aldrich syndrome	XLR
Disorders of RNA synthesis and processing	
Cartilage-hair hypoplasia	AR
Dyskeratosis congenital	XLR, AD, AR
Shwachman-Diamond syndrome	AR
Disorders of metabolism	
Barth syndrome	AR, XLR
Glycogen Storage disease, type 1b	AR
Pearson's syndrome	Mitochondrial
Disorders of vesicular transport	
Chédiak-Higashi syndrome	AR
Cohen syndrome	AR
Griscelli syndrome, type II	AR
Hermansky-Pudlak syndrome, type II	AR
p14 Deficiency	Probable AR

AD = autosomal dominant; AR = autosomal recessive; XLR = X-linked recessive

this case exhibits neutrophilic hyperplasia. Thrombocytopenia and (occasionally) anemia can also accompany hypersplenism.

Pseudo-neutropenia

Pseudo-neutropenia is similar to pseudo-neutrophilia in that it is produced by alterations in the circulating and marginated pools. Pseudo-neutropenia results from the transfer of circulating neutrophils to the marginated neutrophil pool with no change in the total peripheral blood neutrophil pool. This temporary shift is characteristic of some infections with endotoxin production and of hypersensitivity reactions. Because of the selective margination of neutrophils, the total leukocyte count drops and a relative lymphocytosis develops.

False Neutropenia

It is important for the laboratory professional to recognize when neutropenia is a result of laboratory in vitro manipulations of blood. Refer to Table 21-6 ★ for a summary of four in vitro causes of a low neutrophil count. First, neutrophils can (rarely) adhere to erythrocytes when the blood is drawn in EDTA, causing an erroneously low automated white count. If observed on the stained smears, blood can be recollected by finger stick to make manual dilutions and blood smears without utilizing EDTA (Chapter 37). Second, neutrophils disintegrate in blood collection tubes faster than other leukocytes. If there is a delay in testing the blood, the neutrophil count can be erroneously decreased. Third, in some pathologic conditions, the leukocytes are more fragile than normal and can rupture with the manipulations of preparing blood for testing in the laboratory. Finally, the neutrophil count can be falsely decreased if the neutrophils clump together because of the presence of paraproteins (proteins appearing in large quantity because of other pathological conditions).

☑ CHECKPOINT 21-4

How can the correct white cell count be determined when neutrophils clump in the presence of EDTA?

★ **TABLE 21-6** Causes of False Low Neutrophil Counts in Clinical Laboratory Testing

- EDTA-induced neutrophil adherence to erythrocytes
- Disintegration of neutrophils over time prior to testing
- Disruption of abnormally fragile leukocytes during preparation of the blood for testing
- Neutrophil aggregation

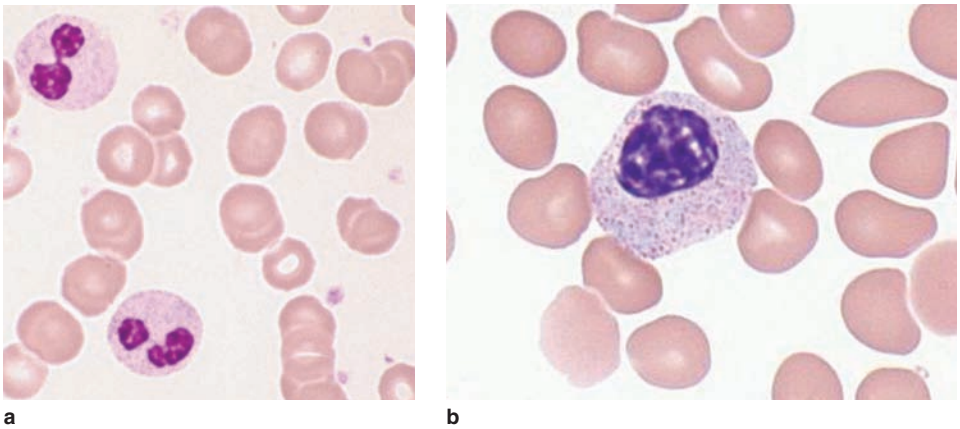
Qualitative or Morphologic Abnormalities

Automated hematology analyzers do not detect or flag neutrophil morphologic abnormalities. Laboratory professionals must microscopically evaluate stained blood smears to identify cytoplasmic and/or nuclear morphologic abnormalities in neutrophils. Cytoplasmic abnormalities are the most common and most cytoplasmic changes (Döhle bodies, toxic granulation, and vacuoles) are reactive, transient changes accompanying infectious states. The correct identification of alterations such as intracellular microorganisms can lead to the prompt diagnosis and treatment of life-threatening infections, whereas recognition of Pelger-Huët or hypersegmented neutrophils can point to the diagnosis of specific conditions that can prove elusive without the morphologic information.

Nuclear Abnormalities

Pelger-Huët Anomaly

Pelger-Huët anomaly (Figure 21-3 ■) is a benign anomaly inherited in an autosomal dominant fashion and occurs in about 1 in 5000 individuals. The neutrophil nucleus does not segment beyond the two-lobed stage and can appear as a single, round nucleus with no segmentation. The presence of excessive coarse chromatin clumping in the nucleus aids in the differentiation of bilobed cells from band neutrophils. The bilobed nucleus has a characteristic dumbbell shape with the two lobes connected by a thin strand of chromatin. Cells with this appearance are often called *pince-nez cells* (French name for the style



a b

■ **FIGURE 21-3** Pelger-Huët anomaly. **(a)** Note pince-nez, or eyeglass-shaped, nuclei. These mature cells could easily be confused for bands if not for the highly clumped chromatin. **(b)** The nucleated neutrophil is the nonsegmented or round nucleus form that can be found (peripheral blood, Wright-Giemsa stain, 1000× magnification).

of eye-glasses without earpieces). Rod-shaped and peanut-shaped nuclei can also be found. Individuals heterozygous for Pelger-Huët anomaly have neutrophil nuclei that are primarily bilobed, whereas the majority of neutrophils in homozygous individuals have a round or oval nucleus with only a few cells having the classic bilobed shape.[17] The cell is functionally normal, and individuals with hereditary Pelger-Huët anomaly do not display increased susceptibility to bacterial infections.[17] The significance of recognizing this anomaly lies in differentiating the benign hereditary defect from a left shift that can occur during infections.

Acquired- or pseudo-Pelger-Huët anomaly can present in myeloproliferative disorders and myelodysplastic states. The neutrophils in pseudo-Pelger-Huët anomaly (sometimes described as pelgeroid) are frequently hypogranular because of a lack of secondary granules, and the nuclei acquire a round rather than a dumbbell shape. The chromatin appears with intense clumping, aiding in differentiation of these mononuclear cells from myelocytes.

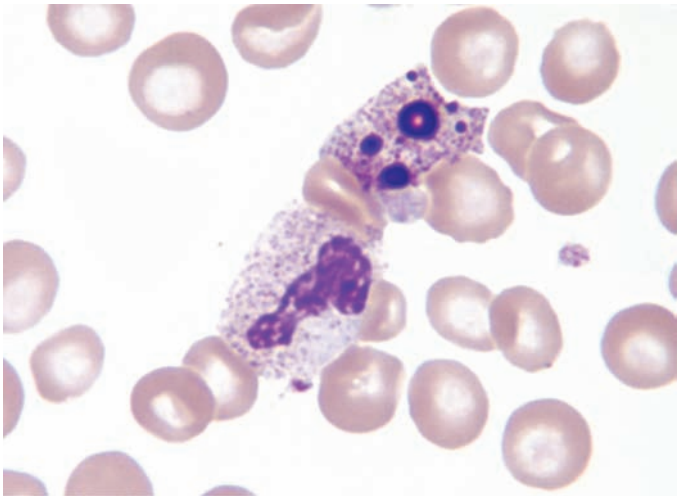
It is particularly important that laboratory professionals distinguish between the Pelger-Huët and pseudo-Pelger-Huët anomalies because the presence of the acquired form can be used to aid in the diagnosis of myelodysplasia and malignancy.[18] To distinguish the two, the inherited Pelger-Huët anomaly usually presents with $<10\%$ of the neutrophils displaying three or more nuclear lobes without the presence of immature myeloid cells (meta- and myelocytes). On the other hand, pseudo-Pelger-Huët neutrophils likely appear in a blood picture with far more than 10% (likely $>50\%$) of the neutrophils showing three or more nuclear lobes and possibly immature myeloid cells.[19]

Hypersegmentation

Larger-than-normal neutrophils with six or more nuclear segments (hypersegmented neutrophils) or five or more neutrophils with five lobes are common and early indicators of megaloblastic anemia.[20] These cells are found together with pancytopenia and macro ovalocytes that typically accompany deficiencies of folate or vitamin B_{12} (Chapter 15). Reported cases of hereditary hypersegmentation of neutrophils, a benign condition, are rarely significant but need to be distinguished from multilobed nuclei that are associated with disease.[21]

Pyknotic Nucleus

Pyknotic, or apoptotic, nuclei (Figure 21-4 ■) are found in dying neutrophils in blood or body fluid preparations. The nuclear chromatin condenses and the segments disappear, becoming smooth, dark-staining spheres. If the nucleus is round, these apoptotic cells can be confused with nucleated erythrocytes.



■ **FIGURE 21-4** The nucleated cell at the top is a dying neutrophil with a pykonotic nucleus. Note the smooth nuclear material that is breaking up (peripheral blood, Wright-Giemsa stain, 1000× magnification).

☑ CHECKPOINT 21-5

Describe the difference between hypersegmented and hyposegmented neutrophils and pyknotic nuclei. In what conditions are each seen?

Cytoplasmic Abnormalities

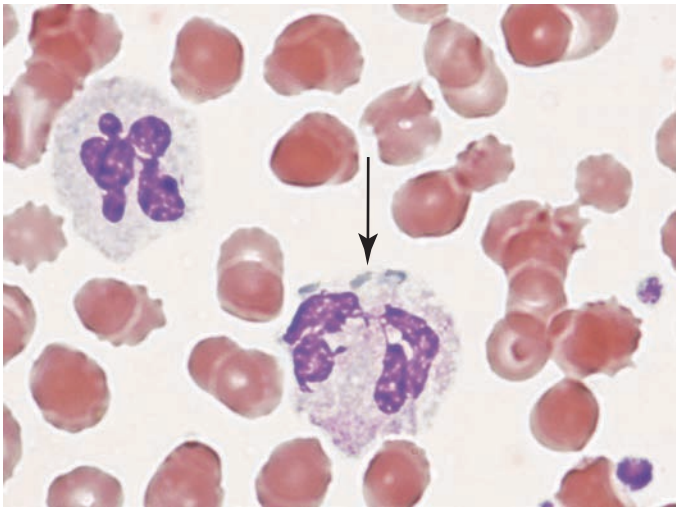
Cytoplasmic inclusions are often found in infectious states and when present, give the health care provider important diagnostic information (Table 21-7 ★). These inclusions are Döhle bodies, toxic granules, vacuoles, and intracellular organisms.

Döhle Bodies

Döhle bodies are light gray-blue oval inclusions in the cytoplasm of neutrophils and eosinophils (Figure 21-5 ■). Found near the periphery of the cell, Döhle bodies are composed of aggregates of rough endoplasmic reticulum. They can be seen in severe infections, burns, and cancer and as a result of toxic drugs. Döhle bodies should be looked for whenever toxic granulation or other reactive morphologic changes are present because they frequently occur together.

★ **TABLE 21-7** Cytoplasmic Inclusions Found in Neutrophils in Infectious Conditions

Inclusion	Morphologic Characteristics	Composition	Associated Conditions
Döhle body	Light gray-blue oval near cell periphery	Rough endoplasmic reticulum (RNA)	Infections, burns, cancer, or inflammatory states
Toxic granules	Large blue-black granules	Primary granules	Same as Döhle body
Cytoplasmic vacuole	Clear, unstained circular area	Open spaces from phagocytosis	Same as Döhle body
Bacteria	Small basophilic rods or cocci	Phagocytized organisms	Bacteremia or sepsis
Fungi	Round or oval basophilic inclusions slightly larger than bacteria	Phagocytized fungal organisms	Systemic fungal infections often in immunosuppressed patients
Morulae	Basophilic, granular; irregularly shaped	Clusters of *Ehrlichia* rickettsial organisms	Ehrlichiosis



■ **FIGURE 21-5** Arrow points to neutrophil with three bluish inclusions (Döhle bodies) (peripheral blood; Wright-Giemsa stain, 1000× magnification).

Döhle bodies are similar in appearance to the cytoplasmic inclusions found in May-Hegglin anomaly (described later in the "May-Hegglin Anomaly" section).

Toxic Granules

Toxic granules are large, deeply stained, blue-black primary granules in the cytoplasm of segmented neutrophils and sometimes in bands and metamyelocytes (Figure 21-6 ■). Primary (nonspecific) granules normally lose their basophilia as the cell matures, so even though about one-third of the granules in the mature neutrophil are primary granules, their presence is not detectable. In contrast, *toxic* primary granules retain their basophilia in the mature neutrophil, perhaps because of a lack of maturation. Additionally, toxic primary granules can become more apparent as the secondary granules are discharged to fight bacteria. Toxic granulation is seen in the same conditions as Döhle bodies. Toxic granules or inclusions can appear as artifacts with increased staining time or decreased pH of the buffer used in the staining process.

Cytoplasmic Vacuoles

Cytoplasmic vacuoles appear as clear, unstained circular areas. Vacuoles probably represent the end stage of phagocytosis (Figure 21-7 ■). They are usually seen in the same conditions as toxic granulation and Döhle bodies. Cytoplasmic vacuoles in neutrophils from a fresh specimen correlate highly with the presence of septicemia.

Vacuoles can also appear as an artifact in smears made from blood that has been collected and stored in EDTA. Vacuoles related to storage are more likely to be smaller and more uniformly dispersed than those in toxic states. Making smears from fresh blood without anticoagulant can eliminate the vacuole artifact.

CASE STUDY (continued from page 392)

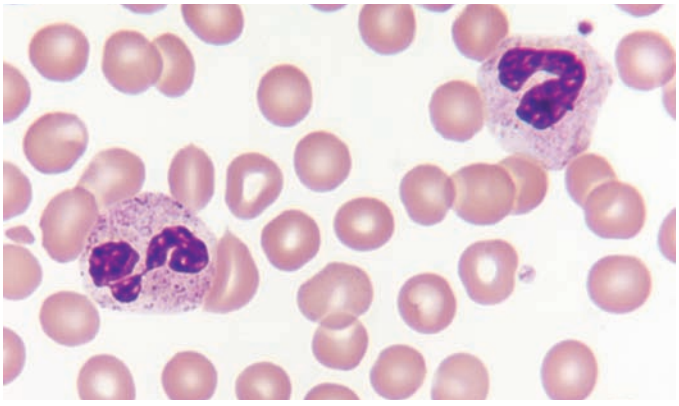
All cultures were negative for bacteria and fungi. On further examination of the blood smear, it was noted that *no* abnormal cytoplasmic features were observed in the neutrophils. However, the nuclei of most of the bands appeared more condensed than normal, and many had two lobes in a dumbbell shape.

3. Given the leukocyte morphology and cultures, what additional condition must now be considered?
4. Explain the clinical significance of the nuclear anomaly described in Dennis.
5. Why is the white cell count elevated?

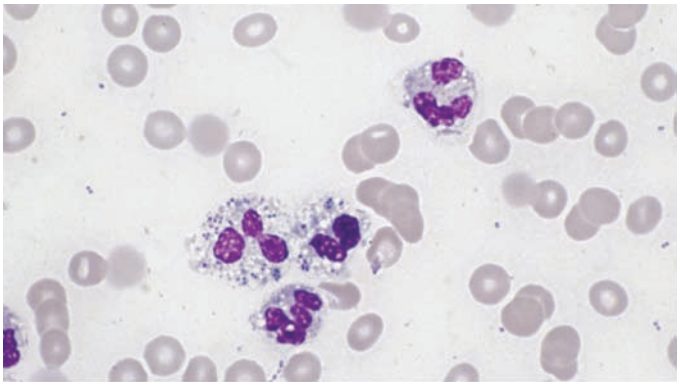
Intracellular Organisms

In most infections, including septicemia, the causative agents are not demonstrable in the peripheral blood. However, microorganisms seen inside neutrophils should always be considered a significant finding, and the physician should be notified immediately (Figure 21-7).

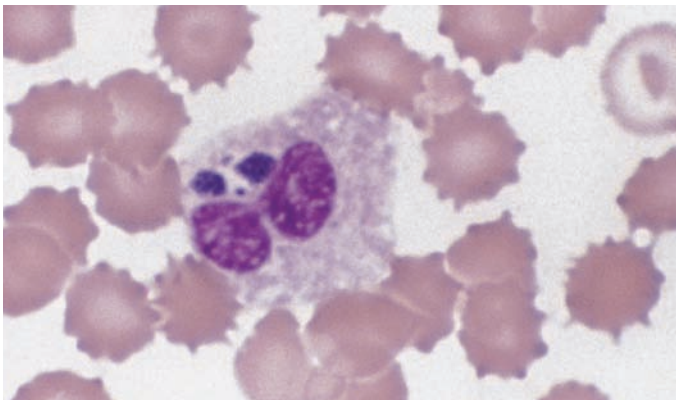
Intracellular *Histoplasma*, or *Candida*, is sometimes found on blood smears from patients with HIV or other severe immunosuppression. Organisms found outside cells must be interpreted with care. On stained blood smears, it is crucial to distinguish whether the microorganisms came from contaminated equipment or stain or are



■ **FIGURE 21-6** A segmented neutrophil and a band neutrophil with toxic granulation (peripheral blood, Wright-Giemsa stain, 1000× magnification).



■ **FIGURE 21-7** Intracellular microorganisms. Note the vacuoles and toxic granulation in the cells (peripheral blood, Wright-Giemsa stain, 1000× magnification).



■ **FIGURE 21-8** Morulae in ehrlichiosis. This segmented neutrophil from a patient with human granulocytic ehrlichiosis contains two dense, basophilic inclusions called *morulae* (peripheral blood, Wright-Giemsa stain, 1000× magnification).

actually present in the patient's blood. Organisms must also be distinguished from other cytoplasmic material and precipitated stain. All bacteria and yeasts stain basophilic with Wright's stain.

In the United States, two known species of *Ehrlichia* infect humans. *E. chaffeensis* infects monocytes and is the causative agent of human monocytic ehrlichiosis (HME), whereas *E. ewingii* infects granulocytes and is the causative agent of human granulocytic ehrlichiosis. *Ehrlichia* sp. are tick-borne, small, obligate intracellular, coccobacilli bacteria. They infect leukocytes where they multiply within phagosomes. The intracellular organisms are pleomorphic, appearing as basophilic, condensed, or loose aggregates of organisms, which tend to appear spherical.

Ehrlichiosis is characterized by high fever, leukopenia, thrombocytopenia, and elevated liver enzymes.[22,23] Most cases of ehrlichiosis occur in April through September when nymphal ticks are most active. The intracellular microcolonies of *Ehrlichia*, called **morulae**, are observed in leukocytes on stained blood films (Figure 21-8 ■). The presence of morulae in leukocytes can be the first diagnostic finding of *Ehrlichia* infection. The leukocyte eventually ruptures and releases the organisms that then infect other leukocytes. The pathogenesis of ehrlichiosis can be related to direct cellular injury by the bacteria or a cascade of inflammatory or immune events.

Confirmation of infection is made through serologic determination of antibody titers using indirect fluorescent antibody (IFA) assays or by identification of DNA sequences by polymerase chain reaction (PCR). Peripheral blood cytopenia is probably the result of sequestration of infected cells in the spleen, liver, and lymph nodes. The bone marrow is usually hypercellular.[24,25]

Anaplasma phagocytophilum also infects neutrophils and is the causative agent of human granulocytic anaplasmosis (HGA). The clinical manifestations and laboratory findings are similar to that of *Ehrlichia* infection, and morulae are observed in the neutrophils.[22]

Inherited Functional Abnormalities

Functional neutrophil abnormalities are almost always inherited and can be accompanied by morphologic abnormalities. It is suggested that granulocyte functional abnormalities be suspected in patients with recurrent, severe infections, abscesses, and delayed wound healing and in antibiotic resistant sepsis. See Table 21-8 ★ for a summary of the functional defects and their clinical features.

Alder-Reilly Anomaly

Alder-Reilly anomaly is an inherited condition characterized by the presence of large purplish granules in the cytoplasm of all leukocytes. Similar morphology is seen in disorders such as Hurler's syndrome and Hunter's syndrome, both of which are lysosomal storage disorders (discussed later in the "Monocyte/Macrophage Disorders" section; see also Figure 21-9 ■). These disorders are characterized by incompletely degraded mucopolysaccharides that accumulate in the lysosomes and appear as large granules.[26] Although the granules can resemble toxic granulation, Alder-Reilly granules can be differentiated because they stain metachromatically with toluidine blue. The granules can appear in lymphocytes and tend to occur in clusters in the shape of dots or commas and are surrounded by vacuoles (Gasser's cells). The inclusions frequently are seen only in cells of the bone marrow, not in the peripheral blood, but in either case, the cells function normally.

★ **TABLE 21-8** Inherited Qualitative Neutrophil Abnormalities

Condition	Morphologic or Functional Defect	Clinical Features
Alder-Reilly anomaly	Large, purplish cytoplasmic granules in all leukocytes Cells function normally	Associated with mucopolysaccharidosis such as Hurler's syndrome
Chédiak-Higashi syndrome	Giant fused granules in neutrophils and lymphs Cells engulf but do not kill microorganisms	Serious, often fatal condition with repeated pyrogenic infections
May-Hegglin anomaly	Blue, Döhle-like cytoplasmic inclusions in all granulocytes Cells function normally	Bleeding tendency from associated thrombocytopenia
Chronic granulomatous disease (CGD)	Defective respiratory burst Cells engulf but don't kill microorganisms	Recurrent infections, especially in childhood
Myeloperoxidase deficiency	Low or absent myeloperoxidase enzyme Cell morphology normal	Usually benign; other bactericidal systems prevent most infections
Leukocyte adhesion deficiency (LAD)	Absence of cell-surface adhesion proteins affecting multiple cell functions Cell morphology normal	Serious condition with recurrent infections and high mortality

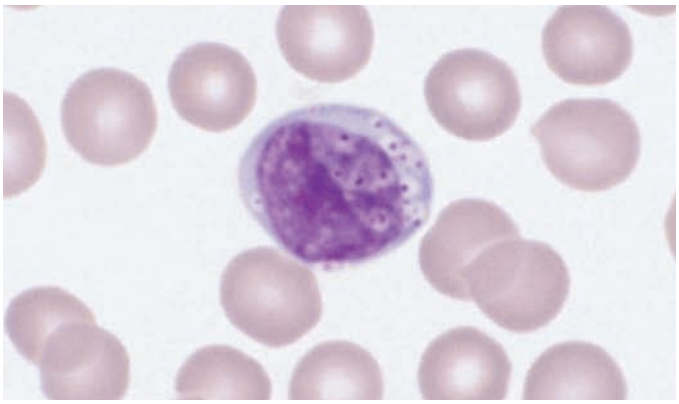


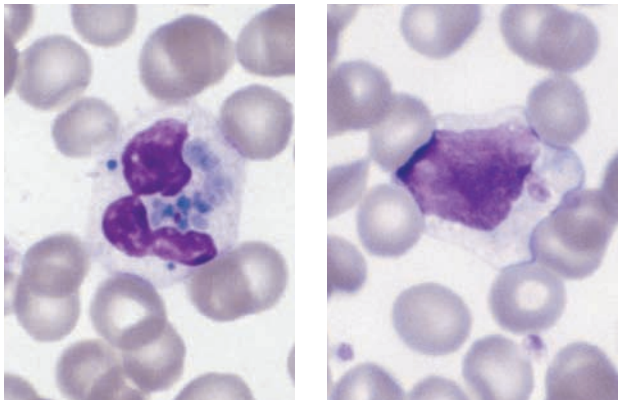
FIGURE 21-9 Lymphocyte from Hurler's disease (mucopolysaccharidoses). Note the halo around the granules (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Chédiak-Higashi Syndrome

Chédiak-Higashi syndrome is a rare autosomal recessive disorder in which death usually occurs in infancy or childhood because of recurrent bacterial infections (Figure 21-10 ■). Giant gray-green peroxidase-positive bodies and giant lysosomes are found in the cytoplasm of leukocytes as well as most granule-containing cells of other tissues. These bodies are formed by fusion of primary, nonspecific and secondary, specific neutrophilic granules. This abnormal fusion of cytoplasmic membranes prevents the granules from being delivered into the phagosomes to participate in killing of ingested bacteria. Neutropenia and thrombocytopenia are frequent complications as the disease progresses. The patients have skin hypopigmentation, silvery hair, and photophobia from an abnormality of melanosomes. Lymphadenopathy and hepatosplenomegaly are characteristic.[27]

May-Hegglin Anomaly

May-Hegglin anomaly is a rare, inherited, autosomal dominant trait in which granulocytes contain inclusions consisting mainly of RNA from rough endoplasmic reticulum, which are similar in appearance



a b

FIGURE 21-10 (a) Neutrophil from Chédiak-Higashi syndrome. (b) Lymphocyte from the same patient as in a. Note the bluish-gray inclusion bodies (peripheral blood, Wright-Giemsa stain; 1000× magnification).

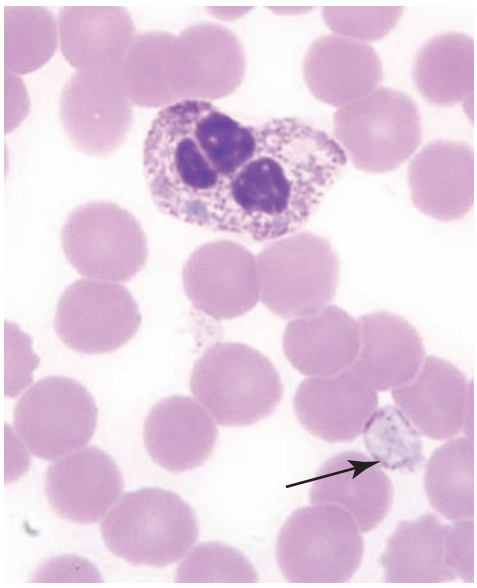


FIGURE 21-11 May-Hegglin anomaly. There is a neutrophil with a Döhle-like structure in the cytoplasm and a large platelet (arrow) (peripheral blood, Wright-Giemsa stain, 1000× magnification).

to Döhle bodies (Figure 21-11 ■). The inclusions can be distinguished from true Döhle bodies because they are usually larger and rounder in shape.[28] Variable thrombocytopenia with giant platelets is characteristic. The only apparent clinical symptom patients can exhibit is abnormal bleeding related to the low platelet count.

Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is an inherited disorder (65% X-linked, 35% autosomal recessive)[29] characterized by defects in the respiratory burst oxidase system. Patients suffer from recurrent, often life-threatening, infections with opportunistic pathogens (bacterial and fungal) that result in the formation of granulomas. CGD can be diagnosed in childhood but in some cases, the onset of symptoms may not occur until early adulthood.

The affected cells in CGD are morphologically normal but cannot generate antimicrobial oxygen metabolites, such as H_2O_2. Therefore, the neutrophils can phagocytize micro-organisms but cannot kill them. Ingested micro-organisms produce hydrogen peroxide as a by-product of oxidative metabolism; those that are catalase-positive are not killed because they destroy the H_2O_2 of their own metabolism. Catalase-positive organisms continue to grow intracellularly where they are protected from antibiotics, whereas catalase-negative organisms kill themselves by generating H_2O_2 that can be used in the phagocytic cell's antimicrobial defense mechanisms (Chapter 7).

The peripheral blood neutrophil count is normal but increases in the presence of infection. Immunoglobulin levels are often increased because of chronic infection.[30] Treatment involves the use of prophylactic antibiotics and early treatment of infections. The nitroblue tetrazolium slide test (NBT) is useful in detecting the abnormal oxygen metabolism of neutrophils in CGD. Neutrophils are mixed with nitroblue tetrazolium and microorganisms. In normal individuals, the leukocytes phagocytize the microorganisms, initiating an increase in oxygen uptake. This process leads to an

accumulation of oxygen metabolites that reduce the NBT to a blue-black compound, which shows in the cell as dark crystals. Neutrophils from individuals with CGD cannot mobilize a respiratory burst, so no dark crystals appear.[31]

The dihydrorhodamine 123 (DHR123) assay using flow cytometry is replacing the NBT test in some laboratories. The DHR123 assay (also referred to as the *neutrophil oxidative burst assay*), incubates granulocytes with bacteria and the dye DHR123.[32] After the neutrophils phagocytize the bacteria, they activate nicotinamide adenine dinucleotide phosphate oxidase and produce reactive oxygen metabolites (the respiratory burst). These metabolites oxidize DHR123 to fluorescent rhodamine 123, which is detected by flow cytometry. In healthy adults, the reference interval for granulocytes with phagocytic activity is 80–100%.

Myeloperoxidase Deficiency

Myeloperoxidase deficiency is, for the most part, a benign autosomal recessive disorder characterized by an absence of myeloperoxidase in neutrophils and monocytes. Although neutrophils use myeloperoxidase in the bactericidal process, an increase in infections is not usually seen in myeloperoxidase deficiency, even in homozygous individuals. The neutrophils are able to utilize alternative antimicrobial systems to kill the microorganisms (although somewhat more slowly) (Chapter 7). However, patients with myeloperoxidase deficiency and diabetes mellitus can experience disseminated fungal infections (usually with *Candida albicans*).[33]

Certain hematology analyzers can detect the presence or absence of myeloperoxidase in a two-stage cytochemical reaction that differentiates cells based on stain and size characteristics. Regarding myeloperoxidase, neutrophils, eosinophils, and monocytes are usually positive and basophils and lymphocytes are negative. The neutrophils in patients with myeloperoxidase deficiency are depicted as large, unstained cells. On peripheral blood smears, the neutrophils appear morphologically normal.

Leukocyte Adhesion Deficiency

Two forms of leukocyte adhesion deficiency are LAD I and LAD II. LAD I is a rare, autosomal recessive disorder characterized by decreased or absent leukocyte cell-surface adhesion proteins (β2-integrins, also termed CD11/CD18 complex) (Chapter 7). Neutrophils from patients with LAD I have multiple functional defects, including impaired adhesion to endothelial cells, chemotaxis, phagocytosis, respiratory burst activation, and degranulation. Because of defective adhesion proteins, the neutrophils cannot adhere to endothelial cells of the blood vessel walls and exit the circulation. In addition, LAD I neutrophils are not able to recognize the presence of the complement C3bi fragment on microorganisms, so phagocytosis is not stimulated. LAD II also is a rare autosomal recessive disorder characterized by failure of endothelial cells to synthesize the ligand for leukocyte L-selectin (Sialy-Le^x). Leukocyte function is normal, but the cells fail to adhere to the vessel wall endothelium and cannot exit the circulation to enter the tissues in host defense responses.

Features of both LAD I and II include recurrent soft tissue bacterial and fungal infections with persistent leukocytosis and granulocytosis due to increased stimulation of the bone marrow. The frequency and severity of the infections in LAD I depends on the amount of CD11/CD18 the cells express. Diagnosis can be made by flow cytometric analysis of neutrophil CD11b levels using a monoclonal antibody.[31] Treatment for both LAD I and II includes prophylactic antibiotics and early, aggressive treatment of infections. Mortality rate in childhood can be high, and bone marrow transplantation is recommended in severe cases of LAD I.

☑ CHECKPOINT 21-6

Explain how you can determine whether toxic granulation and vacuoles in the neutrophils are due to the patient's condition or to artifact.

EOSINOPHIL DISORDERS

Disorders involving exclusively eosinophils are rare. An increase in the circulating number of these cells can indicate a potentially serious condition. Because the lower limit of the reference interval is very low, a decrease is difficult to determine and is probably not significant. Eosinopenia can be seen in acute infections and inflammatory reactions and with the administration of glucocorticosteroids. Glucocorticosteroids and epinephrine inhibit eosinophil release from the bone marrow and increase their margination.[34] Eosinophilia can be classified as clonal (neoplastic, also called primary) or idiopathic (unknown cause and clonality cannot be identified).[35]

Nonclonal (Reactive) Eosinophilia

Eosinophilia refers to an increase in eosinophils above $>0.4 \times 10^9$/L. Reactive eosinophilia appears to be induced by cytokines secreted from T lymphocytes. This type of eosinophilia is polyclonal and the common myeloid progenitor (CMP) cell is normal. Various conditions associated with the cellular immune response (mediated by T lymphocytes) are characterized by eosinophilia including tissue-invasive parasites, allergic conditions, respiratory tract disorders, gastrointestinal diseases, and skin and connective tissue disorders and diseases.[36] Parasites are the most common cause of secondary eosinophilia worldwide. When the eosinophil concentration is high and immature forms are present, the blood picture can resemble that seen in chronic eosinophilic leukemia (Chapter 24). The conditions associated with eosinophilia are listed in Table 21-9 ★.

Tissue invasion by parasites produces an eosinophilia more pronounced than parasitic infestation of the gut or blood. Eosinophils are especially effective in fighting tissue larvae of parasites; they readily adhere to larvae coated with IgG, IgE, and/or complement. Larvae are too large for phagocytosis, so the eosinophil molds itself around the larva to destroy it. Intracellular eosinophilic granules fuse with the eosinophil membrane and expel their contents into the space between the cell and the larva. The granular substances attack the larva wall, partially digesting it.[37,38]

A moderate increase in eosinophils frequently characterizes allergic disorders (asthma, dermatitis, and drug reactions). Large numbers can also be found in nasal discharges and sputum of allergic individuals as well as in the peripheral blood.

★ **TABLE 21-9** Conditions Associated with Quantitative Changes of Eosinophils, Basophils, and Mast Cells

Eosinopenia	Acute infections, inflammatory reactions, administration of glucocorticosteroids
Eosinophilia	
Nonclonal (reactive)	Parasitic infection
	Allergic conditions, especially asthma, dermatitis, and drug reactions
Clonal (neoplastic)	CEL, NOS, eosinophilia with abnormalities of PDGFRA, PDGFRB, or FGFR1, and eosinophilia that occurs in other MPNs
Idiopathic	No known cause
Basopenia	Inflammatory states following immunologic reactions
Basophilia	Immediate hypersensitivity reactions
	Endocrinopathies
	Infectious diseases
	Chronic myeloproliferative disorders, especially CML; chronic basophilic leukemia
Mastocytosis	Immediate hypersensitivity reactions
	Connective tissue disorders
	Infectious diseases
	Neoplastic disorders such as lymphoproliferative diseases and hematopoietic stem cell diseases

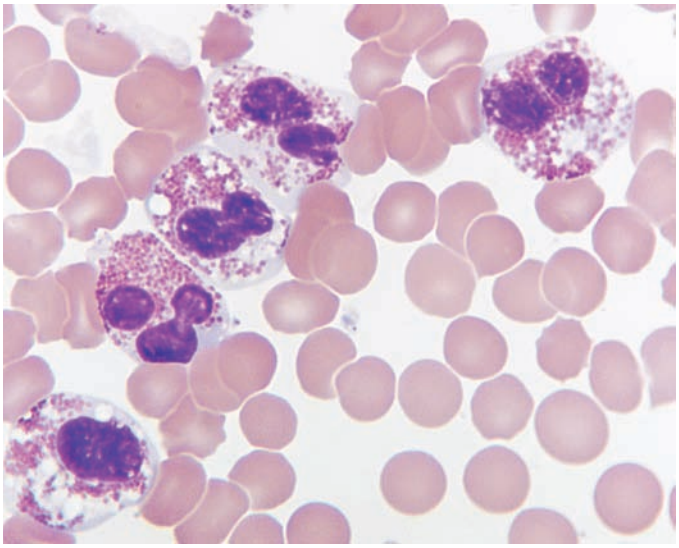
CEL, NOS = chronic eosinophilic leukemia, not otherwise specified; CML = chronic myeloid leukemia; FGFR1 = fibroblast growth factor receptor 1; MPN = myeloproliferative neoplasm; PDGFR = platelet-derived growth factor receptors (PDGFRA, PDGFRB)

Eosinophilia also accompanies a disorder termed pulmonary infiltrate with eosinophilia (PIE) syndrome characterized by asthma, pulmonary infiltrating eosinophils, central nervous system anomalies, peripheral neuropathy, polyateritis nodosa, and local or systemic eosinophilia. This syndrome can be produced by parasitic or bacterial infections, allergic reactions, or collagen disorders. In some cases, no cause can be found.[39]

Clonal (Neoplastic) Eosinophilia

Primary eosinophilia is characterized by a persistent blood eosinophilia of $>1.5 \times 10^9$/L with tissue infiltration (Figure 21-12 ■). It may be a clonal myeloid or lymphoid neoplastic disorder with mutations in genes that code for platelet derived growth factor receptor (PDGFR) or fibroblast growth factor receptor 1 (FGFR1). These disorders are considered myeloproliferative neoplasms and are discussed in Chapter 24. In other cases, the cause for the eosinophilia is unknown and clonality cannot be proven. These disorders are collectively known as idiopathic **hypereosinophilic syndrome (HES)**. The identification of genetic mutations has decreased the number of cases that are considered idiopathic.[36]

Chronic eosinophilia can cause extensive tissue damage as the granules are released from disintegrating eosinophils. In many HES cases, large numbers of circulating eosinophils damage the heart. Charcot-Leyden crystals formed from either eosinophil cytoplasm or granules can be found in exudates and tissues where large numbers of eosinophils migrate and disintegrate. Treatment of HES with corticosteroids, hydroxyurea, and/or α-interferon is sometimes effective in reducing the eosinophil count. HES is most commonly seen in males (>90%) and is rarely seen in children.[35]



■ **FIGURE 21-12** Hypereosinophilic syndrome. Note that the cells are all mature eosinophils (peripheral blood, Wright-Giemsa stain, 1000× magnification).

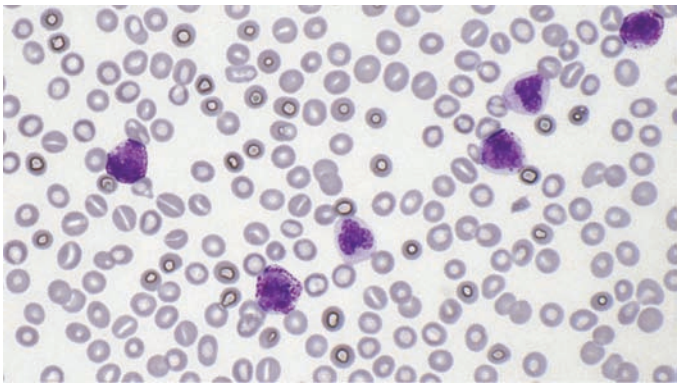
Idiopathic HES must be differentiated from reactive eosinophilias and clonal eosinophilias (such as eosinophilic leukemia). Eosinophilic leukemia usually presents with myeloblasts and eosinophilic myelocytes, whereas idiopathic HES and reactive eosinophilia present with mature eosinophils. In addition, an abnormal clonal chromosome karyotype or molecular mutation suggests eosinophilic leukemia or another clonal variant rather than idiopathic HES or reactive eosinophilia (Chapter 24).

BASOPHIL AND MAST CELL DISORDERS

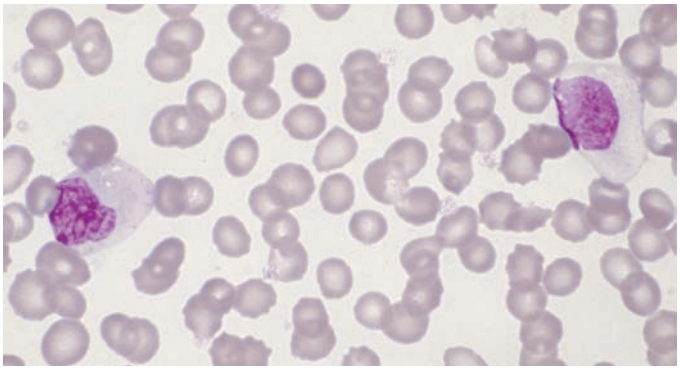
Both basophils and mast cells are important in inflammatory and immediate allergic reactions because they are both able to release inflammatory mediators. Their cytoplasmic granules can be stimulated for release by many of the same mediators. In addition to inflammatory mediators common to both cell types, each has individual unique mediators. Extracellular degranulation of inflammatory mediators can be induced by physical destruction of the cell, chemical substances (toxins, venoms, proteases), endogenous mechanisms (tissue proteases, cationic proteins), and immune mechanisms. The immune mechanisms can be IgE-dependent (IgE binds to high-affinity receptors on these cells) or IgE-independent (triggered by complement fragment C5a binding to C5a receptors on these cells).

The number of basophils and mast cells increases at sites of inflammation. Basophils migrate from the blood by adhering to the endothelium (mediated by several families of adhesion molecules and receptors).[40]

Basophilia refers to an increase in basophils $>0.2 \times 10^9$/L and is associated with immediate hypersensitivity reactions and chronic myeloproliferative disorders (Table 21-9, Figure 21-13 ■). An absolute basophilia is often helpful in distinguishing CML from a leukemoid reaction or other benign leukocytosis. A basophil count exceeding 80% of the total leukocyte population and the absence of the Philadelphia



■ **FIGURE 21-13** Peripheral blood from a patient with CML and 30% basophils (peripheral blood, Wright-Giemsa stain, 1000× magnification).



■ **FIGURE 21-14** Peripheral blood from a patient with reactive monocytosis showing two reactive monocytes (peripheral blood, Wright-Giemsa stain, 1000× magnification).

chromosome and/or *BCR/ABL1* gene rearrangement suggests the diagnosis of acute basophilic leukemia, an extremely rare condition.

A decrease in basophils is even more difficult to establish than eosinopenia. Scanning a blood smear with 100× magnification will reveal a rare basophil in normal individuals. Decreases in basophils are seen in inflammatory states and following immunologic reactions.

Mastocytosis can be found in a number of disorders in which the number of mast cells is increased as much as fourfold in affected tissues (most commonly the skin) and in conjunction with certain neoplastic disorders (Table 21-9). No clinical disorder that involves a decrease in mast cells numbers has been identified; however, long-term treatment with glucocorticoids can lower the number of mast cells.[40]

☑ CHECKPOINT 21-7

Why are the basophil and eosinophil counts important when assessing the benign or neoplastic nature of a disorder?

MONOCYTE/MACROPHAGE DISORDERS

Quantitative disorders are associated with monocytes, whereas qualitative disorders are associated with both monocytes and macrophages. The qualitative disorders are inherited lysosomal storage disorders.

Quantitative Disorders

Monocytosis occurs when the absolute monocyte count is $>0.8 \times 10^9$/L (Figure 21-14 ■). It is seen most often in inflammatory conditions and certain malignancies (Table 21-10 ★). Monocytosis occurring in the recovery stage of acute infections and in agranulocytosis is considered a favorable sign. Monocytes also play an important role in the cellular response against mycobacterium in tuberculosis.

Unexplained monocytosis has been reported to be associated with as many as 62% of all malignancies. Monocytosis can be seen in myelodysplastic states, acute myeloid leukemia, and chronic myeloid leukemia and in approximately 25% of Hodgkin lymphomas. In these conditions, the monocyte is probably a part of a reactive process to the neoplasm rather than a part of the clonal neoplasm itself. Neoplastic proliferation of monocytes occurs in acute monocytic leukemia and acute and chronic myelomonocytic leukemia (Chapters 24, 26).

Monocytopenia refers to a concentration of monocytes $<0.2 \times 10^9$/L and is found in stem cell disorders such as aplastic anemia. Monocytopenia is difficult to establish because of the low normal levels of these cells.

Qualitative Disorders

Lysosomal storage disorders include a large group of inherited disorders. All nucleated cells contain lysosomes used as a part of the cell's recycling system. Lysosomes contain various enzymes (including glucosidases, lipases, proteases, and nucleases) that are involved in degradative processes. Defects in any of these enzymes can lead to the accumulation of either nondegraded substrates or catabolic products that are unable to be transported out of the lysosome. This accumulation can lead to cell dysfunction and pathological phenotypes. Most of these disorders are inherited in an autosomal recessive pattern.[41]

The type of storage material that accumulates (glycoproteins, glycosphingolipids, mucolipids, mucopolysaccharides, etc.) can be used to classify lysosomal storage disorders. Based on this accumulated storage material, there are two main categories: mucopolysaccharidoses (MPS) and lipidoses. Many of these disorders cause

★ **TABLE 21-10** Conditions Associated with Quantitative Changes of Monocytes

Neoplastic	Myelodysplastic/myeloproliferative neoplasms
	Chronic myelomonocytic leukemia
	Juvenile myelomonocytic leukemia
	Chronic myelogenous leukemia
	Acute monocytic, myelomonocytic, and myelocytic leukemias
Reactive	Inflammatory conditions
	Collagen diseases
	Immune disorders
	Certain infections (e.g., TB, syphilis)
Monocytopenia	Stem cell disorders such as aplastic anemia

detectable morphology in granulocytes (see the section "Alder-Reilly Anomaly" earlier in this chapter) and monocytes/macrophages. Disorders based on the presence of abnormal macrophages in hematologic tissue, a common presenting feature, include Gaucher disease, Niemann-Pick disease, and sea-blue histiocytosis (see "Histiocytoses" section). In MPS, the accumulating macromolecules are found primarily in connective tissue.

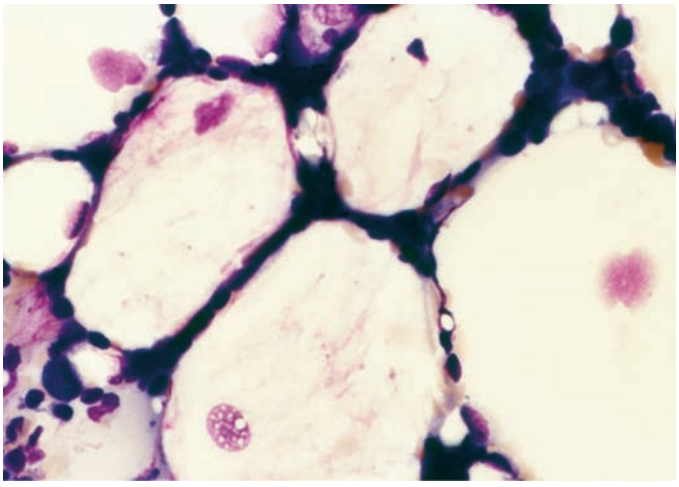
Gaucher Disease

Gaucher (pronounced go-shay) disease is an inherited autosomal recessive disorder characterized by a deficiency of β-glucosidase (an enzyme needed to break down the lipid glucocerebroside). In this disease, the macrophage is unable to digest the stroma of ingested cells, and the lipid glucocerebroside accumulates. The clinical findings (splenomegaly and bone pain) of the disease are related to the accumulation of this lipid in macrophages mainly in the spleen, liver, and bone marrow. The macrophages (Gaucher cells) are large (20–100 mcM [μM]) with small eccentric nuclei, and the cytoplasm appears wrinkled or striated (Figure 21-15 ■).[42] The spleen and liver can become greatly enlarged. Leukopenia, thrombocytopenia, and anemia can occur from sequestration by an enlarged spleen. Diagnosis of Gaucher disease is determined upon detection of insufficient β-glucosidase enzyme activity in peripheral blood leukocytes.[42]

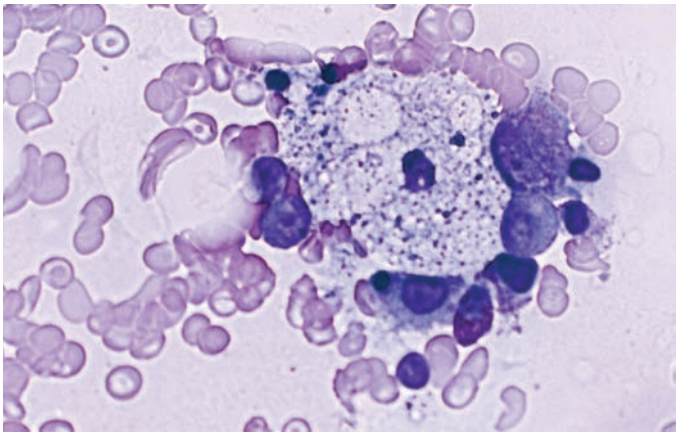
Cells similar to Gaucher cells can be found in the marrow of individuals with a rapid granulocyte turnover, especially in chronic myeloid leukemia. The accumulation of lipid in these disorders does not result from enzyme deficiency but from the inability of the macrophage to keep up with the flow of fat into the cell from the increased cell turnover. Differential diagnosis of Gaucher disease can be confirmed by demonstrating decreased leukocyte β-glucosidase activity, whereas the enzyme level is normal or increased in myeloproliferative disorders.

Niemann-Pick Disease

Niemann-Pick disease includes a group of rare disorders that are related autosomal recessive diseases. Signs of the disease begin in infancy with poor physical development. The spleen and liver are greatly enlarged. The disease is often fatal by 3 years of age. In Niemann-Pick type A and type B disease, the defect is a deficiency of sphingomyelinase (an enzyme needed to break down lipids), resulting in excessive sphingomyelin storage. Macrophages with a foamy appearance are found in lymphoid tissue and the bone marrow (Figure 21-16 ■). The foam cells are large (20–100 mcM) with an eccentric nucleus and globular cytoplasmic inclusions. Leukopenia and thrombocytopenia can occur from increased sequestration by the enlarged spleen, and blood lymphocytes can contain several vacuoles that are lipid-filled lysosomes.[43]



■ **FIGURE 21-15** Gaucher macrophages in bone marrow (peripheral blood, Wright-Giemsa stain, 1000× magnification).



■ **FIGURE 21-16** Macrophage in the bone marrow of a patient with Niemann-Pick disease. Note the foamy cytoplasm with inclusions (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Miscellaneous Lysosomal Storage Disorders

Tay-Sachs disease, Sandhoff disease, and Wolman's disease are inherited lipid storage diseases and Hurler's and Hunter's syndromes are inherited MPS. These disorders are characterized by a deficiency of one or more enzymes that metabolize lipids or mucopolysaccharides. As a result of these enzyme deficiencies, abnormal concentrations of these macromolecules accumulate in the lysosomes of tissue cells. These conditions affect mostly nonhematologic tissue and are often fatal. There are no specific findings in the peripheral blood. Lipid-laden macrophages can be present in the bone marrow.[44]

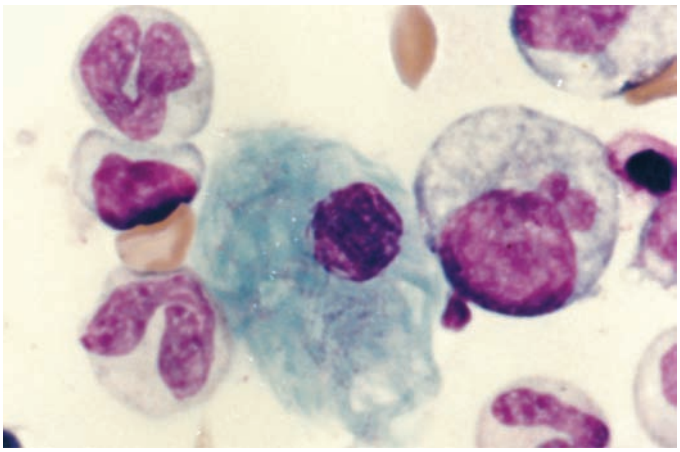
Histiocytoses

Two different types of cells are known as *histiocytes*: Langerhans cells/dendritic cells and monocytes/macrophages. Both cell types are antigen processing and antigen presenting and share a common progenitor. The three classes of histiocytoses are class I—Langerhans cell histiocytoses (LCH); class II—non-Langerhans histiocytoses (non-LCH), which encompasses the histiocytoses of mononuclear phagocytes; and class III—malignant histiocytoses.

The Langerhans cell is an immature dendritic cell usually found in the epidermis, oral and vaginal mucosa, and lungs. They differ from other tissue cells by characteristic racquet-shaped structural inclusions known as *Birbeck granules*. Monocyte/macrophage histiocytes contribute to reactive (in response to an inflammatory stimulus) and

malignant disorders. Reactive macrophage histiocytes can be seen in benign proliferative diseases (xanthoma disseminata, juvenile xanthogranuloma), in nonmalignant hemophagocytic diseases (fulminant hemophagocytic syndrome, histiocytosis with massive lymphadenopathy), and in several of the storage disorders (Gaucher, Niemann-Pick, and sea-blue histiocytosis).

Sea-blue histiocytosis syndrome is a rare inherited disorder characterized by splenomegaly and thrombocytopenia. Sea-blue staining macrophages laden with lipid are found in the liver, spleen, and bone marrow (Figure 21-17 ■). The cell is large (in diameter) with a dense eccentric nucleus and cytoplasm that contains blue or blue-green granules. Considerable variation in clinical manifestations is present, but in most patients, the course of the disease is benign. Sea-blue histiocytes can also be seen in a variety of disorders of lipid metabolism and in association with various other disorders including Niemann-Pick disease, some hematopoietic diseases, and in certain infections.



■ **FIGURE 21-17** Sea-blue histiocyte from bone marrow (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Summary

Leukocytes respond to toxic, infectious, and inflammatory processes to defend the tissues and limit and/or eliminate the disease process or toxic challenge. This can involve a change in leukocyte concentration, most often increasing one or more leukocyte types (depending on the cell's function). Thus, a differential count and the total leukocyte count aid in diagnosis.

Neutrophilia, an increase in neutrophils, most often occurs as a result of a reaction to a physiologic or pathologic process (most commonly, bacterial infection). Tissue injury or inflammation can also cause a neutrophilia. Neutropenia, a decrease in neutrophils, is less commonly encountered. It can be caused by drugs, immune mechanisms, or decreased bone marrow production. Several inherited conditions are characterized by neutropenia and recurrent infections. Morphologic abnormalities of neutrophils can be found in infectious states and are important to identify on stained blood smears. These include Döhle bodies, toxic granulation, and cytoplasmic vacuoles. Other morphologic abnormalities include pince-nez cells found in Pelger-Huët anomaly and morulae found in ehrlichiosis.

Functional and morphologic abnormalities of the leukocyte characterize a number of inherited conditions. Defects in the generation of oxidizing radicals after phagocytosing bacteria characterize chronic granulomatous disease and myeloperoxidase deficiency. Leukocyte adhesion deficiency I (LAD I) is identified by the absence of leukocyte cell-surface adhesion proteins that inhibit the adhesion of neutrophils to endothelial cells so the neutrophils cannot exit the blood. Other rare leukocyte functional abnormalities include Alder-Reilly, Chédiak-Higashi, and May-Hegglin anomalies.

Eosinophils increase in infections with parasites, allergic conditions, hypersensitivity reactions, cancer, and chronic inflammatory states. Basophilia is seen in hypersensitivity reactions, infections, and chronic myeloproliferative disorders, especially chronic myelogenous leukemia. Monocytosis is found in a wide variety of conditions, especially malignancies. Macrophage disorders are commonly associated with a group of lysosomal storage disorders in which these cells are unable to completely digest phagocytosed material.

Review Questions

Level I

1. Which of the following hematologic values would you expect if the peripheral blood smear revealed toxic granulation, Döhle bodies, and vacuoles in neutrophils? (Objectives 1, 2)
   A. WBC: 4.0 $\times$ 10^9/L
   B. the differential shows 15% bands
   C. the differential shows 20% eosinophils
   D. Hb: 10 g/dL; platelets 20 $\times$ 10^9/L
2. The white count in an adult is 2.0 $\times$ 10^9/L. The differential shows 60% segmented neutrophils. Which of the following correctly describes these results? (Objective 6)
   A. normal
   B. leukocytosis and neutrophilia
   C. leukopenia with normal number of neutrophils
   D. leukopenia and neutropenia
3. Which of the following is the most common cause of neutrophilia? (Objective 1)
   A. bacterial infection
   B. acute leukemia
   C. chemotherapy
   D. aplastic anemia
4. Blue-gray oval inclusions composed of RNA near the periphery of neutrophils is a description of: (Objective 3)
   A. toxic granules
   B. Döhle bodies
   C. vacuoles
   D. primary granules
5. Which of the following is a common reason for neutropenia in hospitalized patients? (Objective 6)
   A. chronic myeloproliferative disorders such as CML
   B. chemotherapy and/or radiation treatment for cancer
   C. childbirth
   D. lack of exercise
6. Which of the following causes a false neutropenia? (Objective 7)
   A. EDTA-induced agglutination
   B. bone marrow aplasia
   C. splenomegaly
   D. immune neutropenia
7. Which of the following can be used to distinguish a leukemoid reaction from CML? (Objective 5)
   A. a total WBC count >25 $\times$ 10^9/L
   B. the presence of immature cells of the myeloid lineage
   C. LAP stain
   D. presence/absence of autoantibodies
8. Which of the following is a common cause of eosinophilia? (Objective 10)
   A. parasitic infection
   B. eosinophilic leukemia
   C. CML
   D. acute hemorrhage
9. What substances build up and are ingested by macrophages in qualitative macrophage disorders such as Gaucher disease? (Objective 11)
   A. proteins
   B. carbohydrates
   C. lipids
   D. rough endoplasmic reticulum
10. Distinct, large, unidentified inclusions are found in the cytoplasm of many granulocytes. Select the best course of action. (Objective 9)
    A. Ignore them because they probably are not significant.
    B. Report them as intracellular yeast.
    C. Suspect rare or unusual conditions such as Chédiak-Higashi.
    D. Report them as toxic granulation.

Level II

Use the following case study to answer review questions 1–5.

A patient's white count is 30.0 $\times$ 10^9/L. The differential is as follows:

Segmented neutrophils	54%
Band neutrophils	10%
Metamyelocytes	2%
Lymphocytes	26%
Monocytes	5%
Eosinophils	3%
6 Nucleated RBCs/100 WBCs	

1. Select the additional information most important to assess whether these results are normal or indicate a disease process. (Objective 1)
   A. platelet count
   B. LAP stain
   C. patient history
   D. patient age
2. Which of the following correctly describes the absolute neutrophil count for the patient? (Objectives 1, 12)
   A. normal for both an infant and an adult
   B. neutrophilia for both an infant and an adult
   C. normal for an infant and neutrophilia for an adult
   D. normal for an adult and neutrophilia for an infant
3. Which of the following correctly describes the neutrophil concentration if the patient is an infant? (Objective 1)
   A. pseudo-neutrophilia
   B. leukemoid reaction
   C. leukoerythroblastic reaction
   D. physiologic neutrophilia
4. Which of the following correctly describes the differential if the patient is an adult? (Objective 1)
   A. pseudo-neutrophila
   B. leukoerythroblastic reaction
   C. agranulocytosis
   D. physiologic neutrophilia
5. A leukoerythroblastic reaction can be associated with: (Objectives 1, 2, 12)
   A. chronic granulomatous disease
   B. Chédiak-Higashi syndrome
   C. severe hemolytic anemia
   D. leukocyte adhesion deficiency
6. Alder-Reilly anomaly can be differentiated from toxic granulation by: (Objectives 7, 11)
   A. presence/absence of other toxic features (leukocytosis, toxic granulation, Döhle bodies, vacuoles)
   B. the presence of hypersegmentation in neutrophils
   C. other CBC parameters abnormal (RBC, HCT, or PLT)
   D. patient symptoms related to an allergic reaction
7. May-Hegglin can be differentiated from conditions with toxic Döhle bodies by: (Objectives 7, 11)
   A. presence of thrombocytosis
   B. presence or absence of other toxic features such as toxic granulation
   C. foam cells in the bone marrow
   D. patient history of recent trauma
8. Pelger-Huët anomaly can be differentiated from an increase in band neutrophils by: (Objectives 5, 11)
   A. flow cytometry for the presence of CD11b
   B. abnormal inclusions found in cells other than neutrophils
   C. other CBC parameters abnormal (RBC, HCT, or PLT)
   D. finding all bilobed segmented neutrophils
9. Chédiak-Higashi can be differentiated from intracellular yeasts or morulae by: (Objectives 6, 7, 11)
   A. presence of giant platelets
   B. abnormal inclusions in neutrophils and lymphocytes
   C. other CBC parameters abnormal (RBC, HCT, or PLT)
   D. lack of glucosidase enzyme activity
10. An adult patient's white blood cell count was 10.1×10^9/L, and the absolute neutrophil count was 1.3×10^9/L. The medical laboratory scientist who analyzed the data suspected a false neutropenia and requested a redraw for the patient. Which of the following could have caused the medical laboratory scientist to question the CBC results? (Objective 4)
    A. The blood specimen was drawn 6 days before testing.
    B. The patient's history indicated hypersplenism.
    C. The neutrophil count was too high for an adult.
    D. The patient suffered from congenital neutropenia.
11. Leukemia can be differentiated from infection by: (Objectives 2, 11)
    A. lack of glucosidase enzyme activity in monocytes
    B. a shift to the left in granulocytic cells
    C. a leukocyte alkaline phosphatase stain
    D. flow cytometry for the presence of CD11b
12. A sample with toxic vacuoles can be differentiated from a sample with prolonged storage by: (Objectives 4, 6, 11)
    A. presence of other toxic features (leukocytosis, toxic granulation, Döhle bodies, vacuoles)
    B. abnormal inclusions found in lymphocytes
    C. other abnormal CBC parameters (RBC, HCT, or PLT)
    D. family history

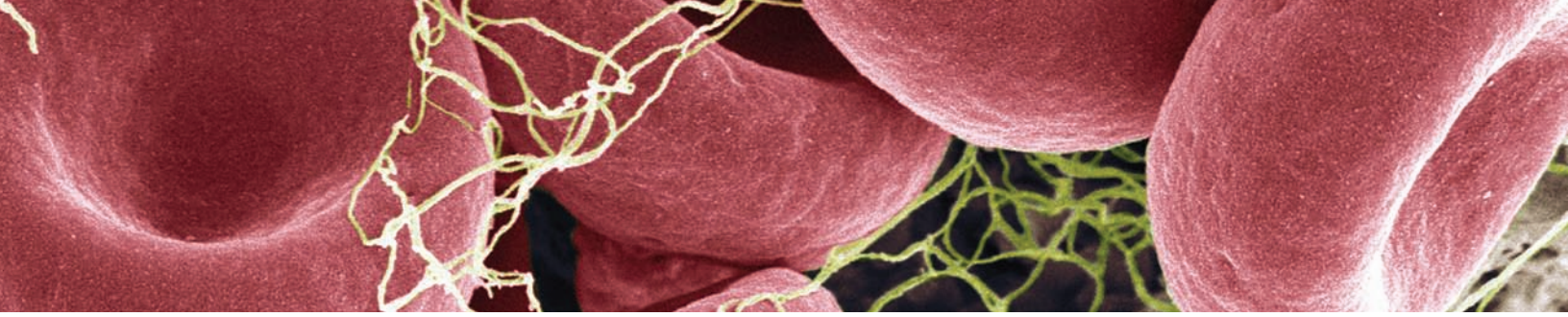
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Kumar V, Abbas AK, Aster JC et al. Diseases of white blood cells, lymph nodes, spleen, and thymus. In: Kumar V, Abbas AK, Aster JC et al., eds. *Robbins and Cotran Pathologic Basis of Disease*, 8th ed. Philadelphia: Saunders Elsevier; 2010:593–94.
2. Dale DC. Chapter 65. Neutropenia and neutrophilia. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*. 8th ed. New York: McGraw-Hill; 2010.
3. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. *Immunology*. 2008;125:281–88.
4. Alves-Filho JC, de Freitas A, Spiller F et al. The role of neutrophils in severe sepsis. *Shock*. 2008;30(suppl 1):3–9.
5. Dinauer MC, Coates TD. Disorders of phagocyte function and number. In: Hoffman R, Furie B, Benz EJ Jr et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:687–90.
6. Mukhopadhyay S, Mukhopadhyay S, Banki K et al. Leukemoid reaction: a diagnostic clue in metastatic carcinoma mimicking classic Hodgkin lymphoma. *Immunology*. 2004;128:1445–47.
7. Vusirikala M. Supportive care in hematologic malignancies. In: Greer JP, Foerster J, Lukens JN et al., eds. *Wintrobe's Clinical Hematology*, 11th ed. Philadelphia: Lippincott Williams & Wilkins; 2004:1997–2044.
8. Cupps TR, Fauci AS. Corticosteroid-mediated immunoregulation in man. *Immunol Rev*. 1982;65:133–55.
9. Sampson AP. The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy*. 2000;30(suppl 1):22–27.
10. Liesveld JL, Lichtman MA. Acute myelogenous leukemia. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1277–331.
11. Freifeld AG, Bow EJ, Sepkowitz KA et al. Infectious Diseases Society of America. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2011;52:e56–93.
12. Dale DC, Welte K. Cyclic and chronic neutropenia. *Cancer Treat Res*. 2011;157:97–108.
13. Hestdal K, Welte K, Lie SO et al. Severe congenital neutropenia: abnormal growth and differentiation of myeloid progenitors to granulocyte colony-stimulating factor (G-CSF) but normal response to G-CSF plus stem cell factor. *Blood*. 1993;82:2991–97.
14. Boxer LA, Newburger PE. A molecular classification of congenital neutropenia syndromes. *Pediatr Blood Cancer*. 2007;49:609–14.
15. Capsoni F, Sarzi-Puttini P, Zanella A. Primary and secondary autoimmune neutropenia. *Arthritis Res Ther*. 2005;7:208–14.
16. Dale DC. Nonmalignant disorders of leukocytes. In: Dale DC, Federman DD, eds. *Hematology: VII. ACP Medicine Online*. New York: WebMD; 2004:1–16.
17. Speeckaert MM, Verhelst C, Koch A et al. Pelger-Huët anomaly: a critical review of the literature. *Acta Haematol*. 2009;121:2029.
18. Cunningham JM, Patnaik MM, Hammerschmidt DE et al. Historical perspective and clinical implications of the Pelger-Huët cell. *Am J Hematol*. 2009;84:116–19.
19. Constantino BT. Pelger-Huët anomaly—morphology, mechanism, and significance in the peripheral blood film. *Lab Med*. 2005;36(2):103–107.
20. Nathan, DG, Oski, AF. *Hematology of Infancy and Childhood*, 4th ed. Philadelphia: WB Saunders; 1998:388–96.
21. Bohinjec J. Myelokathexis: chronic neutropenia with hyperplastic bone marrow and hypersegmented neutrophils in two siblings. *Blut*. 1981;42:1916.
22. Thomas RJ, Dumler JS, Carlyon JA. Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and *Ehrlichia ewingii* ehrlichiosis. *Expert Rev Anti Infect Ther*. 2009;7:709–22.
23. Wormser GP, Dattwyler RJ, Shapiro ED et al. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. *Clin Infect Dis*. 2006;43:1089–134.
24. Bakken JS, Dumler JS. Human granulocytic ehrlichiosis. *Clin Infect Dis*. 2000;31:554–60.
25. Hamilton KS, Standaert SM, Kinney MC. Characteristic peripheral blood findings in human ehrlichiosis. *Mod Pathol*. 2004;17:512–17.
26. Van Hoof F. Mucopolysaccaridosis and mucolipidoses. *J Clin Path*. 1974;27:64–93.
27. Dinauer MC, Coates TD. Disorders of phagocyte function and number. In: Hoffman R, Furie B, Benz EJ Jr et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:698–700.
28. Bennet JS. Hereditary Disorders of Platelet Function. In: Hoffman R, Furie B, Benz EJ Jr et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2009:2134–15.
29. Assari T. Chronic granulomatous disease; fundamental stages in our understanding of CGD. *Med Immunol*. 2006;5:4.
30. Seger RA. Modern management of chronic granulomatous disease. *Br J Haematol*. 2008;140:255–66.
31. Borregaard N, Boxer LA. Disorders of neutrophil function. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010 (Chapter 66).
32. Crockard AD, Thompson JM, Boyd NA et al. Diagnosis and carrier detection of chronic granulomatous disease in five families by flow cytometry. *Int Arch Allergy Immunol*. 1997;114:144–52.
33. Cech P, Stalder H, Widmann JJ et al. Leukocyte myeloperoxidase deficiency and diabetes mellitus associated with Candida albicans liver abscess. *Am J Med*. 1979;66:149–53.
34. Wardlaw AJ. Eosinophils and their disorders. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:897–914.
35. Tefferi A, Patnaik MM, Pardanani A. Eosinophilia: secondary, clonal and idiopathic. *Br J Haematol*. 2006;133:468–92.
36. Kremyanskaya M, Ackerman SJ, Butterfield JH et al. Eosinophilia, eosinophil-associated diseases, chronic eosinophil leukemia, and the hypereosinophilic syndromes. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier Churchill Livingstone; 2013:1077–94.
37. Powell N, Walker MM, Talley NJ. Gastrointestinal eosinophils in health, disease and functional disorders. *Nat Rev Gastroenterol Hepatol*. 2010;7:146–56.
38. Linch SN, Gold JA. The role of eosinophils in non-parasitic infections. *Endocr Metab Immune Disord Drug Targets*. 2011;11:165–72.
39. Oermann CM, Panesar KS, Langston C et al. Pulmonary infiltrates with eosinophilia syndromes in children. *J Pediatr*. 2000;136:351–58.
40. Galli SJ, Metcalfe DD, Arber DA et al. Basophils and mast cells and their disorders. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:915–32.
41. Grabowski GA. Phenotype, diagnosis, and treatment of Gaucher's disease. *Lancet*. 2008;372:1263–71.
42. Jmoudiak M, Futerman AH. Gaucher disease: pathological mechanisms and modern management. *Br J Haematol*. 2005;129:178–88.
43. Zimran A, Elstein D. Lipid storage diseases. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1065–77.
44. McGovern MM, Desnick RJ. Abnormalities of the monocyte-macrophage system: lysosomal storage diseases. In: Greer JP, Foerster J, Lukens JN et al., eds. *Wintrobe's Clinical Hematology*, 11th ed. Philadelphia: Lippincott Williams & Wilkins; 2004:1819–25.



22 Nonmalignant Lymphocyte Disorders

SUE S. BEGLINGER, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Identify the infectious agent and describe the clinical symptoms associated with infectious mononucleosis.
2. Describe and recognize the reactive morphology of lymphocytes found in infectious mononucleosis.
3. Relate the heterophile antibody test to infectious mononucleosis.
4. Given a differential and leukocyte count, calculate an absolute lymphocyte count and differentiate it from a relative lymphocyte count.
5. Identify reactive cell morphology associated with viral infections, and compare it to normal lymphocyte morphology.
6. Describe clinical symptoms of disorders in which a leukocytosis is caused by lymphocytosis.
7. State the complications associated with cytomegalovirus (CMV) infections.
8. Identify absolute and relative lymphocytopenia and lymphocytosis, and list conditions associated with these abnormal counts.
9. Explain the pathophysiology of HIV infections, and describe how it affects lymphocytes.
10. Describe the abnormal hematological findings associated with AIDS.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Assess and resolve/explain conflicting results from peripheral blood morphology and serologic tests in suspected Epstein-Barr virus (EBV) infection.
2. Describe the pathophysiology of infectious mononucleosis.
3. Assess and correlate antibody titers found in infectious mononucleosis with respect to the various EBV viral antigens.
4. State the pathophysiology of toxoplasmosis infections, and explain the resulting lymphocytosis.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

5. Differentiate benign lymphocytic leukemoid reactions from neoplastic lymphoproliferative disorders by laboratory results and characteristics of cell morphology.
6. Define the pathophysiology of CMV infection, and give clinical findings associated with it.
7. In *Bordetella pertussis* infection, propose the cause of lymphocytosis and recognize laboratory features associated with it.
8. Assess a patient case using the AIDS case surveillance criteria of the Centers for Disease Control and Prevention (CDC) and recommend appropriate laboratory testing.
9. Explain the cytopenia, identify the defect, and recognize the laboratory features in congenital qualitative disorders of lymphocytes.
10. Evaluate a case study from a patient with a lymphoproliferative disorder and conclude from the medical history and laboratory results the most likely diagnosis for the disorder.
11. Identify the cytokine associated with hyper IgE syndrome (HIES) and explain its role in the pathophysiology of the disorder.

Key Terms

Acquired immune deficiency syndrome (AIDS)
Advanced HIV disease (AHD)
Bordetella pertussis
Cytomegalovirus (CMV)
Epstein-Barr virus (EBV)
Heterophile antibody
Hyper IgE syndrome (HIES)
Immunosuppressed
Infectious mononucleosis
Lymphocytic leukemoid reaction
Opportunistic organisms
Persistent polyclonal B-cell lymphocytosis (PPBL)
Reactive lymphocytosis
Severe combined immunodeficiency (SCID) syndrome
Toxoplasmosis
Viral load

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review the following concepts before starting this unit of study:

Level I

- Describe normal and reactive lymphocyte morphology and identify the distinguishing characteristics of T and B lymphocytes. (Chapter 8)
- Calculate absolute cell counts and summarize the relationship between the hematocrit and hemoglobin. (Chapters 7, 10)
- Define *antigen* and *antibody*, describe their roles in infectious and noninfectious diseases, and summarize the immune response. (Chapter 8)

Level II

- Describe the process of T and B lymphocyte differentiation and the function of each subtype, summarize the structure and function of each of the immunoglobulins, and describe the immune response. (Chapter 8)
- Describe the structure and function of the hematopoietic organs and tissue. (Chapter 3)
- Explain the principle and the application of direct antiglobulin tests in diagnosis of immune-mediated anemia. (Chapter 19)

CASE STUDY

We will refer to this case study throughout the chapter.

Heidi, a 54-day-old female, was admitted to the hospital because of recurrent respiratory distress and failure to gain weight. She was born prematurely at 35 weeks gestation by urgent Cesarean section. Her mother was immune to rubella and had negative serologic tests for syphilis.

Consider why the lymphatic system should be evaluated in this child and the possible etiology of repeated respiratory problems.

OVERVIEW

This chapter discusses benign conditions associated with quantitative and qualitative alterations in lymphocytes and describes infectious mononucleosis and other acquired disorders characterized by lymphocytosis. A discussion of lymphocytopenia and immune deficiency states, both acquired and congenital, follows. Emphasis is on the laboratory features that allow the diseases to be diagnosed and differentiated from neoplastic lymphoproliferative disease.

INTRODUCTION

Evidence of disease, especially infectious disease, can be observed by finding abnormal concentrations of lymphocytes and/or reactive lymphocytes on a peripheral blood smear. This finding helps direct

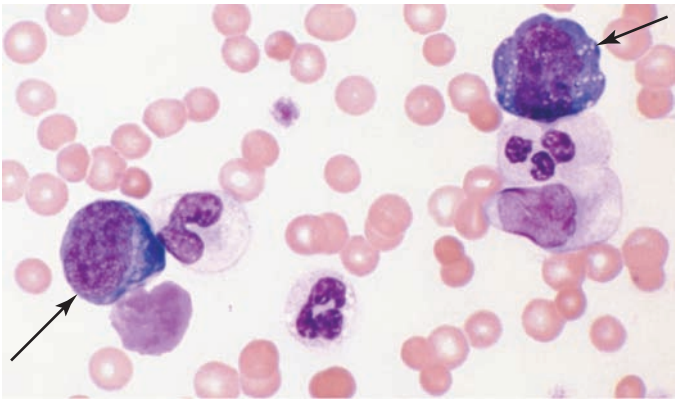
the physician's subsequent workup of the patient and aids in the initiation of appropriate therapy. Most disorders affecting lymphocytes are acquired and are characterized by a reactive lymphocytosis. Some acquired disorders result in a lymphocytopenia that can compromise the function of the immune system. In acquired disorders affecting lymphocytes, the change in lymphocyte concentration and morphology is a reactive process. In contrast, in congenital disorders involving lymphocytes, the primary defect is within the lymphocytic system.

LYMPHOCYTOSIS

Lymphocytosis, an increase in lymphocytes, can result from a relative or absolute increase in lymphocytes. Absolute lymphocytosis occurs in adults when the lymphocyte count exceeds 4.8×10^9/L and relative lymphocytosis is present when the lymphocyte differential exceeds 35–45% (differs with race). An absolute lymphocytosis can occur without relative lymphocytosis, and a relative lymphocytosis can occur without absolute lymphocytosis. The lymphocyte concentration in children is normally higher than in adults and varies with the child's age (Table B, inside front cover). Lymphocytosis can occur with or without a leukocytosis.

Lymphocytosis is usually a self-limiting, reactive process that occurs in response to an infection or inflammatory condition. Both T and B lymphocytes are commonly affected, but their function remains normal. Occasionally, viral infections can cause functional impairment of the lymphocytes, yielding both a qualitative disorder and quantitative changes.

Once lymphocytes have been stimulated by an infection or inflammatory condition, they enter various states of activation, resulting in a morphologically heterogeneous population of cells on stained blood smears (Figure 22-1 ■). These activated cells can appear large with irregular shapes and cytoplasmic basophilia, and granules and vacuoles can be seen. The nuclear chromatin usually becomes more dispersed (Chapter 8). Cells with these activation features are commonly referred to as *reactive* or *atypical lymphocytes*. The preferred term is *reactive lymphocytes* because these cells are not, in fact, "atypical." Occasionally, intense proliferation of lymphoid elements in the lymph nodes and spleen occurs, causing lymphadenopathy and splenomegaly, respectively.



■ **FIGURE 22-1** Forms of stimulated lymphocytes from a case of infectious mononucleosis. There are two immunoblasts (one o'clock and eight o'clock) and a reactive lymph (three o'clock) (peripheral blood, Wright-Giemsa stain, 1000× magnification).

T lymphocytes normally compose about 60–80% of peripheral blood lymphocytes. Thus, increases in the concentration of T lymphocytes are more likely to cause changes in the relative lymphocyte count than are increases in B lymphocytes. Absolute lymphocytosis is not usually accompanied by leukocytosis except in infectious mononucleosis, *Bordetella pertussis* infection, cytomegalovirus infection, and lymphocytic leukemia. A relative lymphocytosis secondary to neutropenia that occurs in a variety of viral infections is more commonly found. The absolute lymphocyte count is calculated as:

$$\%\ \text{Lymphocytes (decimal form)} \times \text{WBC count } (\times 10^9/\text{L}) = \text{Absolute cell count } (\times 10^9/\text{L})$$

It is important to differentiate benign conditions associated with lymphocytosis from neoplastic or malignant lymphoproliferative disorders (Table 22-1 ★). The presence of heterogeneous reactive lymphocytes, positive serologic tests for the presence of specific antibodies against infectious organisms, and absence of anemia and thrombocytopenia favor a benign diagnosis. This chapter includes a discussion of the more common disorders associated with a reactive lymphocytosis.

★ **TABLE 22-1** Conditions Associated with Lymphocytosis

Benign (Nonmalignant) Conditions	Neoplastic Conditions
• Infectious mononucleosis	• Acute lymphoblastic leukemia
• *B. pertussis* infection	• Chronic lymphocytic leukemia
• Toxoplasmosis	• Hairy cell leukemia
• Persistent polyclonal B-cell lymphocytosis	• Heavy chain disease
• Viral infections	• Multiple myeloma
Chicken pox	• Waldenström's macroglobulinemia
Coxsackie virus	
Cytomegalovirus infection	
Measles	
Mumps	
Roseola infantum	
Infectious hepatitis	
• Chronic infections	
Tertiary syphilis	
Congenital syphilis	
Brucellosis	
• Endocrine disorders	
Thyrotoxicosis	
Addison's disease	
Panhypopituitarism	
• Convalescence of acute infections	
• Immune reactions	
• Inflammatory diseases	

CASE STUDY (continued from page 409)

Admission CBC on Heidi was WBC: 7.6 × 10^9/L; Hct: 0.55 L/L; Plt: 242 × 10^9/L; and differential: 84% segs, 2% bands, 4% lymphocytes, 8% monocytes, and 2% eosinophils.

1. Does this patient have a leukocytosis or leukopenia?
2. Does this patient have an abnormal lymphocyte count? Explain.

Infectious Mononucleosis

Infectious mononucleosis is a self-limiting lymphoproliferative disease caused by infection with **Epstein-Barr virus (EBV)**. It usually affects young adults; the peak age for infection is 14–24 years. Infection in children from lower-income groups usually occurs before 4 years of age; in more affluent populations, peak infection incidence occurs during adolescence. About 80–90% of adults have had exposure and possess lifelong immunity. Not considered highly contagious, the disease is transmitted through direct contact with saliva that contains EBV.

Cellular immunity (the function of T lymphocytes) is important in limiting the viral growth of EBV-infected B lymphocytes. Immune-compromised individuals are thus at increased risk of serious infection. EBV-associated B-cell tumors and lymphoproliferative syndromes can occur in transplant patients and patients with acquired immune deficiency syndrome (AIDS).[1] These patients have severe T lymphocyte immunodeficiency. Lacking a T lymphocyte response, male children with the rare X-linked lymphoproliferative disorder (XLP) are unable to limit EBV infection of B lymphocytes. As a result, a fatal polyclonal B lymphocyte proliferation occurs.[2]

EBV Pathophysiology

EBV attaches to a receptor on the B lymphocyte membrane designated CD21, which is the receptor for the C3d complement component.[1] The virus infects resting B lymphocytes as well as epithelial cells of the oropharynx and cervix. Binding of the virus to the B lymphocyte activates the cell and induces the expression of the activation marker CD23, which is the receptor for a B lymphocyte growth factor.[3] Once internalized, the virus is incorporated into the B lymphocyte genome, instructing the host cell to begin production of EBV proteins. These viral proteins are then expressed on the cell membrane. Thus, EBV-infected cells express markers of activated B lymphocytes as well as viral markers. The viral genome is maintained in the lymphocyte nucleus and passed on to the cell's progeny. This results in EBV-immortalized B lymphocytes and possible latent infection.

A complex, multifaceted cellular immune response controls acute EBV infection. In the first week of illness, a polyclonal increase in immunoglobulins occurs. During the second week, however, the number of immunoglobulin-secreting B lymphocytes decreases because of the action of CD8+ T lymphocytes. Activated cytotoxic T cells that resemble activated natural killer cells are present early in the disease. Cytotoxic T lymphocytes inhibit the activation and proliferation of EBV-infected B lymphocytes and participate in the cell-mediated immune response. The majority of the reactive lymphocytes seen in the peripheral blood are these CD8+ cytotoxic T lymphocytes.

★ **TABLE 22-2** Summary of Typical Clinical and Laboratory Findings in Infectious Mononucleosis

Clinical Findings	Laboratory Findings
• Lymphadenopathy	• Leukocytosis
• Fever	• Lymphocytosis
• Lethargy	• Elevated C-reactive protein
• Sore throat	• >20% reactive (atypical) lymphocytes
• Splenomegaly	• Immunoblasts present
• Headache	• Heterophile antibodies present
	• Positive antigen tests for EBV
	• Elevated aminotransferases (ALT, AST)

Clinical Findings

Early symptoms include lethargy, headache, fever, chills, sore throat, nausea, and anoxia. The classic triad presentation of symptoms are fever, pharyngitis, and lymphadenopathy (Table 22-2 ★).[2] Children younger than 10 years of age are often asymptomatic but have reactive lymphocytes and elevated C-reactive protein (CRP). Increases in liver aminotransferases are generally found in children >10 years of age.[4] The cervical, axillary, and inguinal lymph nodes are commonly enlarged. Splenomegaly occurs in 50–75% of these patients, and hepatomegaly occurs in about 25%. Occasionally, jaundice develops. Hematologic complications that can occur during or immediately after the disease include autoimmune hemolytic anemia, thrombocytopenia, agranulocytosis, and (very rarely) aplastic anemia. The disease is usually self-limiting, resolving within a few weeks.

Laboratory Findings

Hematologic findings provide important clues for diagnosis in infectious mononucleosis. Serologic tests can confirm the diagnosis.

Peripheral Blood

During active viral infection, an intense proliferation of lymphocytes occurs within affected lymph nodes. The leukocyte count is usually increased (12–25 × 10^9/L) primarily because of an absolute lymphocytosis. Lymphocytosis begins about 1 week after symptoms appear, peaks at 2–3 weeks, and remains elevated for 2–8 weeks. Lymphocytes usually constitute >50% of the leukocyte differential with >20% reactive lymphocytes. The platelet count is often mildly decreased; concentrations of <100 × 10^9/L are rare.

Various forms of reactive lymphocytes can be found in the peripheral blood (Figure 22-2 ■). Typical cells are irregular in shape and have large amounts of spreading cytoplasm with irregular basophilia. Other reactive cells can have deep blue cytoplasm and vacuoles. Immunoblasts are usually present early in the disease. Plasmacytoid lymphocytes and an occasional plasma cell also can be found. When present, immunoblasts should be distinguished from leukemic lymphoblasts to prevent a misdiagnosis. The chromatin pattern of leukemic lymphoblasts is usually finer than the reticular chromatin of immunoblasts. In addition, immunoblasts generally have a lower N:C ratio with more abundant, sometimes vacuolated, cytoplasm. Another important criterion that helps differentiate infectious mononucleosis from leukemia is the morphologic heterogeneity of the lymphocyte

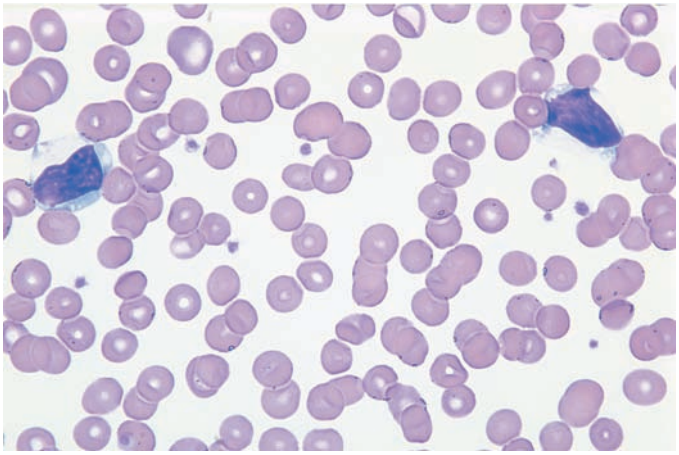


FIGURE 22-2 Two reactive forms of lymphocytes from a case of infectious mononucleosis. Note the cytoplasmic vacuoles and cytoplasmic basophilia and irregular shapes (peripheral blood, Wright-Giemsa stain, 1000× magnification).

population, characteristic of viral infections, whereas leukemia usually has a relatively homogeneous cell population.

Other diseases associated with a reactive lymphocytosis can mimic the blood picture of infectious mononucleosis. These include cytomegalovirus infection, viral hepatitis, and toxoplasmosis.

Bone Marrow

Bone marrow aspirations in EBV infection are not indicated, but when performed show hyperplasia of all cellular elements except neutrophils.

Serologic Tests

Serologic tests (tests based on antigen–antibody reactions) are used to differentiate this disease from similar more serious diseases (e.g., diphtheria, hepatitis). The blood of patients with infectious mononucleosis contains greatly increased concentrations of transient **heterophile antibodies** that agglutinate sheep or horse erythrocytes but are not specific for EBV[5,6] (a heterophile antibody reacts with antigens common to multiple species). Antibodies specific for EBV can be identified by first absorbing the nonspecific heterophile antibodies from patient serum with guinea pig antigen and testing the absorbed serum with horse erythrocytes. The infectious mononucleosis IgM antibodies react with horse erythrocytes. Positive agglutination of horse erythrocytes by treated serum indicates EBV infection. A negative result usually indicates infection by some other virus. On occasion, the level of antibody is not yet high enough to be detected; therefore, the test should be repeated a week later if patient symptoms continue.

Rapid, specific, and sensitive slide agglutination or solid phase immunoassay tests are available to determine the presence of infectious mononucleosis heterophile antibodies. The infected individual also produces antibodies specific for the viral capsid antigen (VCA) of EBV at various stages of infection, which can be detected earlier than the heterophile antibodies. VCA-IgM rises first, followed by VCA-IgG, which when present, signals the development of immunity.[6] Antibodies to EBV nuclear antigens (EBNA) rise during early convalescence and persist together with VCA-IgG (Table 22-3 ★). Thus, the presence of EBNA excludes an acute infection.

★ **TABLE 22-3** Antibodies to EBV Found in Infectious Mononucleosis

Stage of Infection	Heterophile Antibodies	VCA-IgM (titer)	VCA-IgG (titer)	EBNA (titer)
Acute (0–3 months)	Present	>1:160	>1:60	Not detected
Recent (3–12 months)	Present	Not detected	>1:160	>1:10
Past (>12 months)	Present	Not detected	>1:40	>1:40

VCA = viral capsid antigen; EBNA = EBV nuclear antigen

A patient with all clinical manifestations and peripheral blood findings of infectious mononucleosis occasionally does not have a positive heterophile test (heterophile-negative syndrome). In 10–20% of adult cases and 50% of children younger than 10 years of age, the test is negative in the presence of EBV infection. In other cases, the heterophile-negative syndrome is caused by a non-EBV viral infection. The most likely causative agent is cytomegalovirus. Antibody responses might not be detected in **immunosuppressed** individuals (those in whom the immune response is suppressed either naturally, artificially, or pathologically).[6] Viral load testing can be important in immune-compromised patients who develop fulminant liver disease when the disease process is not self-limiting.[7]

Other laboratory tests can be abnormal, depending on the presence or absence of complications. Hepatitis of some degree is common and can be a severe complication. An increase in both direct and indirect bilirubin fractions and an increase in serum liver enzymes are common findings in the presence of hepatitis. A rare complication of infectious mononucleosis is hemolytic anemia. The anemia appears to be caused by cold agglutinins directed against the erythrocyte I-antigen.

Therapy

Because the disease is normally self-limited, therapy is supportive. Bed rest is recommended if fever and myalgia are present. Strenuous exercise should be avoided for several weeks, especially if splenomegaly is present. Antibiotics are not useful except in the presence of secondary infections. Antiviral drugs may be helpful for immune-compromised individuals.[7]

☑ CHECKPOINT 22-1

A patient with lymphocytosis showing reactive lymphocyte morphology with large, basophilic cells, fine chromatin, and a visible nucleolus has a negative infectious mononucleosis serologic test. What is a possible cause for this altered lymphocyte morphology?

Toxoplasmosis

Toxoplasmosis is the result of infection with the intracellular protozoan *Toxoplasma gondii (T. gondii)*. This obligate intracellular parasite can multiply in all body cells except erythrocytes. Infections are often asymptomatic but pose a significant risk to the fetus of a pregnant woman. Transplacental infection can cause abortion, jaundice, hepatosplenomegaly, chorioretinitis, hydrocephalus, microcephaly, cerebral calcification, and mental retardation. Acquired infection can sometimes cause symptoms resembling infectious mononucleosis. *T. gondii* is acquired by ingestion of oocysts from cat feces or from inadequately cooked meat. Toxoplasmosis seropositivity is more common in rural than urban children.[8] Molecular tests for *T. gondii* DNA are available.

Laboratory findings assist in diagnosis. Leukocytosis with a relative lymphocytosis or (more rarely) an absolute lymphocytosis, and an increase in reactive lymphocytes are present. Most reactive cells are morphologically similar to lymphoblasts or lymphoma cells. The heterophile antibody test is negative. Biopsy of lymph nodes shows a reactive follicular hyperplasia and can play an important role in diagnosis. Diagnosis of an active infection is confirmed by seroconversion and a rising titer of antibodies to *T. gondii*. Immunologically compromised hosts have a more severe infection. The most common hematologic complication is hemolytic anemia, which can be severe.

Cytomegalovirus

Infection with the herpes-group virus **cytomegalovirus (CMV)** can be the result of congenital or acquired infection. Infection in neonates occurs when virus from the infected pregnant woman crosses the placenta and infects the fetus. The newborn can demonstrate jaundice, microcephaly, and hepatosplenomegaly. However, only about 10% of infected infants exhibit clinical evidence of the disease. The most common hematologic findings in neonates are thrombocytopenia and hemolytic anemia.

Acquired infection is spread by close contact, blood transfusions, and sexual contact. The disease occurs in immunosuppressed individuals, in patients with malignancy, in patients after massive blood transfusions, and in previously healthy adults. It is the most common viral infection complicating tissue transplants and is a significant cause of morbidity and mortality in immunocompromised patients.[9] However, recent advances in treatment with antiviral drugs and immunosuppressive therapy have dramatically decreased the incidence of CMV complications in organ transplantation.[10] Infected adults present with symptoms similar to those of infectious mononucleosis except that pharyngitis is absent. Many cytomegalovirus infections are subclinical or cause mild flulike symptoms.

Laboratory findings include a leukocytosis with an absolute lymphocytosis. Many lymphocytes show a reactive morphology, but the heterophile antibody test is negative. Hepatic enzymes are usually abnormal. Diagnosis is confirmed by demonstrating the virus in the urine or blood using a viral DNA (molecular) assay or by a rise in the cytomegalovirus antibody titer (except in immunocompromised patients).

CMV is thought to infect neutrophils, which serve as a means of transporting the virus to other body sites. The virus seems to suppress cell-mediated immune function and induce formation of autoantibodies that have lymphocytotoxic properties.[11] A decrease in circulating CD4+ T helper lymphocytes and an increase in CD8+ cytotoxic T lymphocytes occur.

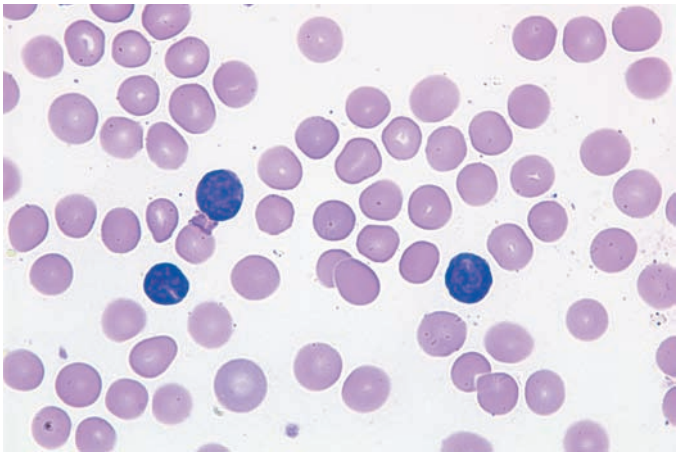
The Reactive Lymphocytosis Process

A **reactive lymphocytosis** process, previously called *infectious lymphocytosis*, is a reactive immune response associated with several common viruses that infect children. This is most often found with adenovirus and coxsackie virus infections.[12] Leukocytosis and lymphocytosis occur in the first week of illness and subsequently return to normal. The lymphocytes are small and appear normal rather than reactive.

Bordetella pertussis

Infection with ***Bordetella pertussis (B. pertussis)***—whooping cough—causes a blood picture very similar to that of reactive viral lymphocytosis (Figure 22-3 ■). The leukocyte count typically rises to $15–25 \times 10^9$/L but can reach 50×10^9/L. The rise in leukocytes is caused by an absolute lymphocytosis of T, B, and natural killer (NK) lymphocytes along with increases in neutrophils and monocytes. Granulocytes may show toxic changes. The lymphocytes are small cells with condensed chromatin and indistinct nucleoli.[13,14]

Laboratory diagnostic methods include culture, serology, immunophenotyping, and PCR.[13] Culture is the gold standard and is specific but not sensitive. The advantage of culture is that it allows antibiotic sensitivity testing and epidemiological typing. Serologic diagnosis is based on demonstrating antipertussis toxin antibodies. It is preferred for older children and adults who present after a prolonged coughing illness. PCR for *B. pertussis* DNA is most sensitive in detecting infection when used during the first 3 weeks of cough[15] and is most useful in acutely ill infants.



■ **FIGURE 22-3** Lymphoproliferation resulting from *B. pertussis* (whooping cough). Note the numerous small lymphocytes with condensed chromatin. This self-limiting peripheral blood picture must be distinguished from that of CLL (peripheral blood, Wright-Giemsa stain, 1000× magnification).

A pertussis toxin secreted by the bacteria causes an accumulation of lymphocytes in the blood by recruiting lymphocytes into the peripheral circulation and blocking their migration back into lymphoid tissue. The toxin interferes with expression of L-selectin (CD62L) on all leukocytes, but lymphocytes are particularly sensitive. Decreased cellularity of the lymph nodes accompanies the rapid peripheral lymphocytosis. The ratio of CD4 to CD8 lymphocytes remains normal.[14]

The incidence of whooping cough infections continues to rise, suggesting that pertussis immunizations given in childhood do not yield lasting immunity. Studies show that 20–30% of adults with a cough that lingers 6–8 weeks are positive for *B. pertussis*. The increased incidence of infections also may be due to increased sensitivity of PCR detections.[15] The infections cause a lingering cough in adults, and passing it on to infants can be fatal.[16]

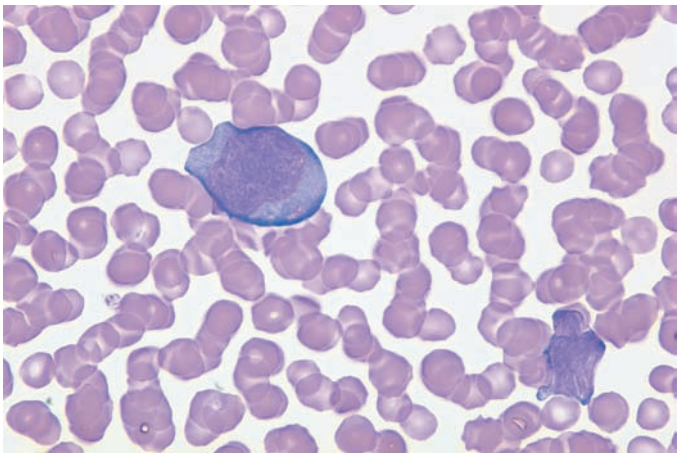
Persistent Polyclonal B-Cell Lymphocytosis

Persistent polyclonal B-cell lymphocytosis (PPBL) is a rare disorder found primarily in the female adult smoker. This population is found to have a polyclonal expression of lymphocytes. However, there often is evidence of an HLA-DR7 haplotype, partial insertion of chromosome 3, i(3q), and multiple IgH/Bcl-2 rearrangements that suggest PPBL could lead to a malignant process.[17] The disorder is often asymptomatic and found by chance on a routine blood analysis. Symptoms can include fever, fatigue, weight loss, recurrent chest infections, or generalized lymphadenopathy. Hematologic findings are normal except for lymphocytosis and the presence of binucleated lymphocytes. There is a polyclonal increase in serum IgM but low IgG and IgA levels. Bone marrow examination reveals lymphocytic infiltrates. Studies that follow patients with PPBL show cases have evolved into a variety of malignant lymphoproliferative disorders including non-Hodgkin lymphoma, diffuse large cell lymphoma, splenic marginal zone lymphoma, and other clonal solid tumors.[17]

Other Conditions Associated with Lymphocytosis

Table 22-1 lists other conditions accompanied by a reactive lymphocytosis. An increased relative lymphocyte count with the presence of reactive or immature-appearing lymphocytes (**lymphocytic leukemoid reaction**) characterizes the lymphocytic reactions in these disorders. An absolute lymphocytosis is found occasionally. Many of the lymphocytes are large, reactive cells with deep blue cytoplasm and fine chromatin, and may show cytoplasmic vacuoles (Figure 22-4 ■). Reactive cells are usually nonclonal T lymphocytes and large granular lymphocytes. In some viral infections, lymphocytopenia and neutropenia precede lymphocytosis. As the infection subsides, plasmacytoid lymphocytes can be found (Figure 22-5 ■).

In some cases, a lymphocytic leukemoid reaction resembles chronic lymphocytic leukemia (CLL) (Chapter 29). Bone marrow aspiration, however, shows minimal (if any) increase in lymphocytes in a lymphocytic leukemoid reaction. In contrast to CLL, lymphadenopathy and splenomegaly are usually absent. In addition, patients with a lymphocytic leukemoid reaction are usually young, whereas CLL patients are usually older adults.

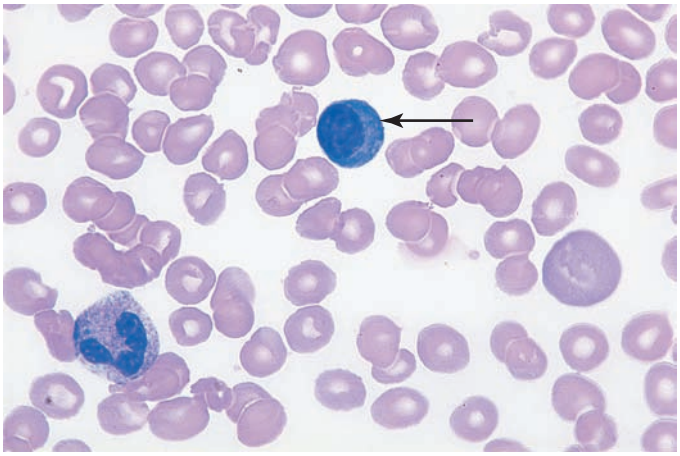


■ **FIGURE 22-4** An immunoblast found in a patient with a viral infection. This cell must be distinguished from a leukemic lymphoblast (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Plasmacytosis

Plasma cells are not normally found in the peripheral blood and constitute <4% of the cells in the bone marrow. Most plasma cells are found in the medullary cores of lymph nodes although they can occasionally be found in the peripheral blood with intense stimulation of the immune system. This can occur in some viral and bacterial infections such as rubeola, infectious mononucleosis, toxoplasmosis, syphilis, and tuberculosis. Circulating plasma cells also can be found in disorders associated with elevated gamma globulin such as multiple myeloma, skin diseases, cirrhosis of the liver, collagen disorders, and sarcoidosis.

Normal plasma cell morphology is included with the discussion of lymphocyte morphology in Chapter 8. A morphologic variation of the reactive plasma cell is called the *flame cell*, named for its reddish-purple cytoplasm. A glycoprotein produced in the rough endoplasmic



■ **FIGURE 22-5** A plasmacytoid lymphocyte. Note the deep basophilic cytoplasm and eccentric nucleus. These cells are associated with infectious states. Note also the neutrophil with toxic granulation (peripheral blood, Wright-Giemsa stain, 1000× magnification).

reticulum (RER) causes the red tinge, and the presence of ribosomes causes the purple tinge. These cells contain more immunoglobulin than normal plasma cells. Flame cells have been associated with IgA multiple myeloma but are now recognized in a variety of immune pathologies and occasionally are found in normal bone marrow.

Additional plasma cell changes, more often associated with the malignant disorders plasma cell leukemia and multiple myeloma, are discussed in Chapter 28, "Mature Lymphoid Neoplasms." These include Mott cells, Russell bodies, and Dutcher bodies.

LYMPHOCYTOPENIA

Some disorders are associated with a lymphocytopenia, which occurs in adults when the absolute lymphocyte count is $<1.0 \times 10^9$/L. In children younger than 5 years old, the lower limit of the reference interval varies, but it is higher than in adults (Table B, inside front cover). In general, lymphocyte counts less than $<2 \times 10^9$/L are abnormal in this population. When the lymphocyte count is decreased, an impaired ability to mount an immune response is possible, resulting in immunodeficiency.

Lymphocytopenia results from decreased production or increased destruction of lymphocytes, changes in lymphocyte circulation patterns, and other unknown causes (Table 22-4 ★).[18] Corticosteroid therapy causes a sharp drop in circulating lymphocytes within 4 hours. The decrease is caused by sequestration of lymphocytes in the bone marrow. Values return to normal within 12–24 hours after cessation of therapy. Acute inflammatory conditions, including viral and bacterial infections, also can be associated with a transient lymphocytopenia. Carcinoma of the breast and stomach with an associated lymphocytopenia is a poor prognostic sign. Systemic lupus erythematosus is frequently associated with a lymphocytopenia presumably caused by autoantibodies produced against these cells. Chemotherapeutic alkylating drugs for malignancy, such as cyclophosphamide, cause the death of T and B lymphocytes in both interphase and mitosis. Malnutrition is the most common cause of lymphocytopenia. Starvation causes thymic involution and depletion of T lymphocytes. Both congenital and acquired immune deficiency disorders are associated with lymphocytopenia.

★ **TABLE 22-4** Conditions Associated with Lymphocytopenia

- Malnutrition
- Disseminated neoplasms
- Connective tissue disease (e.g., systemic lupus erythematosus)
- Hodgkin's disease
- Chemotherapy
- Radiotherapy
- Corticosteroids
- Acute inflammatory conditions
- Chronic infection (e.g., tuberculosis)
- Congenital immune deficiency diseases
- Acquired immune deficiency diseases
- Acute and chronic renal disease
- Stress

Irradiation causes a prolonged suppression of lymphocyte production. CD4+ T helper lymphocytes are more sensitive to radiation than are CD8+ T lymphocytes. It appears that small daily fractions of radiation are more damaging to lymphocytes than periodic large doses. With periodic radiation, the lymphocytes can renew during periods of nonradiation. Aggressive treatment of hematologic malignancies with chemotherapeutics or ionizing radiation can lead to immunodeficiency by depleting short-lived, antigen-activated B lymphocytes.

☑ CHECKPOINT 22-2

Why is lymphocytopenia a concern if there is no accompanying leukopenia?

CASE STUDY (continued from page 411)

Heidi weighed 2 pounds 1 ounce at birth. Tests for CMV and toxoplasma infections were negative. At 4 days of age, she was transferred to a special facility for feeding and growth monitoring. She developed a diaper rash that failed to respond to many measures. No thrush was found. At 44 days, she developed pneumonia from coagulase-negative *staphylococci* that responded to antibiotics. Her WBC count was 12.3×10^9/L with a differential of segs 42%, bands 5%, lymphocytes 1%, monocytes 28%, eosinophils 23%, and basophils 1%.

3. What is the absolute lymphocyte count?
4. What possible causes exist for these opportunistic infections?

Immune Deficiency Disorders

Immune deficiency disorders are characterized by impaired function of one or more of the components of the immune system, T, B, or NK lymphocytes. In some disorders, the numbers of these cells also is reduced. This results in an inability to mount a normal adaptive immune response. These disorders are characterized clinically by an increase in infections and neoplasms and can be subgrouped as acquired or congenital.

Acquired immune deficiency syndrome (AIDS) is an infectious disorder characterized by lymphocytopenia. Because of the frequency with which it is encountered in the clinical hematology laboratory, it is discussed in more detail next and is followed by a discussion of the congenital immune deficiency disorders.

Acquired Immune Deficiency Syndrome

Acquired immune deficiency syndrome (AIDS) is a highly lethal immune deficiency disease first described in 1981.[19] The disease is caused by infection with a retrovirus, human immunodeficiency virus type I (HIV-1) or type 2 (HIV-2). Patients experience weight loss, fever, lymphadenopathy, thrush, chronic rash, and intermittent diarrhea. In the pre-antiretroviral era, patients infected with HIV had a poor

prognosis. Current highly active antiretroviral treatment (HAART) protocols provide effective therapy to reduce viral replication and delay the onset of AIDS for people infected with HIV.[20]

Not all individuals infected with the HIV virus (HIV-positive individuals) have the clinical condition known as AIDS. AIDS is defined by the occurrence of repeated infections with multiple **opportunistic organisms** and an increase in malignancies, especially Kaposi's sarcoma, in individuals infected with HIV. Transmission of the virus is through sexual intercourse or contact with blood and blood products. Infants born to HIV-infected mothers are at increased risk for perinatal HIV infection.

Surveillance HIV Case Definition

In 1982, the U.S. Centers for Disease Control and Prevention (CDC) developed a case definition of AIDS for surveillance purposes. It is revised periodically as more data are collected about the disease.[21] The 2014 revision of Surveillance Case Definition for HIV infection describes the clinical criteria and "opportunistic illnesses" for presumptive evidence of HIV infection[22] (Table 22-5 ★) and standardizes data collection for improved therapeutic treatment. Symptoms of recurrent infections with opportunistic infections warrant suspicion of HIV infection.

The revised criteria apply to adults and children over 18 months of age and combined the HIV classification system and AIDS case definition into a single case definition for *HIV infection*. This revised case definition for HIV infection requires laboratory-confirmed evidence of HIV infection. The Council of State and Territorial Epidemiologists (CSTE), in cooperation with pediatricians, also described the criteria for a negative diagnosis for children ≤18 months old born to HIV-infected mothers. The child does *not* meet the criteria for HIV/AIDS diagnosis if antibody tests or virologic tests are negative after 4–6 months of age and the child does not have an AIDS-defining condition. The reader is directed to the primary source for details.[22] Almost all children born to HIV-infected mothers have anti-HIV IgG antibodies at birth because of placental transmission of maternal antibodies. However, only 15–30% of the children are infected with HIV. Thus, anti-HIV IgG tests in this population are not reliable until after 18 months of age. In these cases, tests for the detection of HIV nucleic acid performed after the infant is at least one month of age are almost always positive if the virus is present.[22]

In addition, the 2008 CDC surveillance HIV data allow for staging of HIV infections based on immunophenotyping of CD4 lymphocytes and clinical conditions (Table 22-6 ★). For surveillance purposes, the staging of disease illness only progresses; it is never reclassified as a less severe stage.

The World Health Organization (WHO) uses similar criteria for diagnosing HIV infection as the CDC, but WHO splits the CDC stage 2 into two separate stages: stage 2 and stage 3. Those patients with decreased lymph counts but not requiring treatment are classified as stage 2, and those patients with decreased CD4+ T lymphs and conditions that require antiretroviral therapy for **advanced HIV disease (AHD)** as stage 3. WHO classifies patients with both clinical conditions and the diagnostic lymphopenia as stage 4 AIDS.[22]

Pathophysiology

The main etiologic agent of AIDS in the United States is the retrovirus, HIV-1. A related but immunologically distinct virus, HIV-2, is endemic to regions of West Africa. The virus selectively infects helper T lymphocytes by binding to both the CD4 protein that is part of the T-cell receptor (TCR), and the chemokine receptor CCR5.[23] Once in the cell, HIV sheds its viral coat and uses reverse transcriptase to make a DNA copy of the viral RNA. Viral DNA is then integrated into the host cell DNA where the virus replicates. Viral infection of these CD4 lymphocytes causes cytolysis, rapid selective depletion of this lymphocyte subset, and eventually lymphocytopenia. Monocytes and macrophages also have the CD4 and CCR5 proteins and are infected but not destroyed by the virus. Rare individuals are homozygous for a 32-base pair deletion in the gene for CCR5 (*CCR5* delta 32), resulting in lack of protein expression on the cell surface. These individuals are resistant to HIV infections.[23]

Cell-mediated immunity and humoral immunity are abnormal. Cell-mediated immunity declines as CD4+ T-lymphocyte-helper function for monocytes, macrophages, and other T lymphocytes declines.[24] Humoral responses are exaggerated with polyclonal B lymphocyte proliferation, increased immunoglobulin production, and hypergammaglobulinemia.[23] Although the B lymphocytes have a poor response to mitogens in vitro, they secrete immunoglobulin spontaneously. This suggests the B lymphocytes are already activated and unable to react to further in vitro stimulation.

★ **TABLE 22-5** Opportunistic Illnesses in HIV Infection

- Bacteria infections, multiple or recurrent[a]
- Candidiasis, bronchi, trachea, or lungs
- Candidiasis, esophageal
- Cervical cancer, invasive
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (>1 month's duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)
- Cytomegalovirus retinitis (with loss of vision)
- Encephalopathy, HIV related
- Herpes simplex: chronic ulcer(s) (>1 month's duration) or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, disseminated or extrapulmonary
- Isosporiasis, chronic intestinal (>1 month's duration)
- Kaposi's sarcoma
- Lymphoma, Burkitt
- Lymphoma, immmunoblastic (or equivalent term)
- Lymphoma, primary, of brain
- *Mycobacterium avium* complex or *M. kansasii* (disseminated or extrapulmonary)
- *Mycobacterium tuberculosis*, any site (pulmonary or extrapulmonary)
- *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary
- *Pneumocystis carinii* pneumonia
- Pneumonia, recurrent
- Progressive multifocal leukoencephalopathy
- *Salmonella* septicemia, recurrent
- Toxoplasmosis of brain
- Wasting syndrome due to HIV

[a]Only among children <6 years old.
From Appendix, *MMWR Recomm Rep*, 63(RR-03):1–10, April 2014.

★ **TABLE 22-6** CDC and WHO Staging of HIV Infection for Adolescents and Adults

CDC Staging	WHO Staging	AIDS-Defining Condition(s)	CD4 + T Lymph Count[a] (cells/mcL)	CD4 + T Lymph (percentage[a] of total lymphocytes)
Stage 1 HIV infection	Stage 1 HIV infection	None	CD4+ lymphs ≥500	CD4+ lymphs ≥26
Stage 2 HIV infection	Stage 2 HIV infection	None	CD4+ lymphs 200–499	CD4+ lymphs 14–25
	Stage 3 HIV infection (AHD)	Clinical conditions requiring retroviral treatment	CD4+ lymphs 200–499	CD4+ lymphs 14–25
Stage 3 AIDS	Stage 4 AIDS	Clinical condition present	CD4+ lymphs <200	CD4+ lymphs <14

[a]Staging requires either CD4+ T lymph count or percent CD4+ T lymphs of total lymphocytes.
Adapted from *Appendix, MMWR Recomm Rep*, 63(RR-03):1–10, April 2014.

Laboratory Findings

Multiple hematologic abnormalities, including leukopenia, lymphocytopenia, anemia, and thrombocytopenia, are found in AIDS (Table 22-7 ★). Leukopenia is usually related to lymphocytopenia, although neutropenia also can be present.[25] Lymphocytes can include reactive forms. Mild to moderate normocytic, normochromic anemia is present in the majority of HIV-infected individuals and worsens as the disease progresses.[26] Inflammatory cytokines may play a role in suppressing erythropoiesis in a manner similar to that found in anemia of chronic disease (ACD) (Chapter 12). Macrocytosis (MCV>100 fL) occurs in up to 70% of patients 2 weeks after receiving zidovudine.[27] Antierythrocyte antibodies can be found in up to 20% of patients with hypergammaglobulinemia. These antibodies react like polyagglutinins and cause a positive direct antiglobulin test (DAT, Coombs' test). Immune thrombocytopenia, indistinguishable from idiopathic thrombocytopenic purpura (ITP), is common (Chapter 33). Iron studies are similar to those found in ACD with low serum iron and TIBC and increased serum ferritin.

Disease Monitoring

The severity of CD4 lymphocytopenia and concentration of plasma HIV-1 RNA copies correlate with the severity of disease.[20] The normal CD4:CD8 ratio in peripheral blood is about 2:1. In AIDS, this ratio reverses progressively and permanently because of destruction of the CD4+ T lymphocytes. The CD4+ T lymphocyte count is performed at initial diagnosis and measured periodically to monitor disease progression. AIDS (CDC stage 3 HIV infection) is defined by a CD4+ lymphocyte count of <200/mcL (μL) or a CD4+ lymphocyte concentration <14% of total lymphocytes.[22] In addition, the **viral load** also is monitored by measuring the number of copies of HIV-1 RNA.[22]

Therapy

No cure for AIDS currently exists. Treatment with zidovudine (azodothymidine, AZT) and protease inhibitors lengthen the time between HIV seropositivity and the onset of AIDS.[28] Improved antiretroviral treatments have postponed the onset of AIDS indefinitely in some patients.[20]

In 2008, a medical group in Germany reported an apparent cure for an HIV-1 positive patient who had received a bone marrow transplant (BMT) for acute myelogeous leukemia.[29] The donor was homozygous for the *CCR5* delta 32 mutation, and the transplanted hematologic cells were resistant to viral infection by HIV-1. Although widely covered in both the scientific and popular (lay) literature, this information has not made a significant impact to date on the treatment strategies for most AIDS patients. BMT is not an option for most AIDS patients, due to both the logistic improbability of finding an HLA-compatible/*CCR5* delta 32 homozygous donor and the cost and medical risks involved with the procedure. However, it has sparked interest in the possibility of a type of gene therapy in which the CCR5 gene is modified in an effort to prevent the HIV virus from being able to infect hematologic cells.[30]

Immediate evaluation and treatment for HIV exposure has proven to be very effective in eliminating or significantly minimizing the risk of HIV infection and AIDS. Health care workers with occupational exposure (e.g., needle-stick injury) should receive immediate antiretroviral therapy.[31] CDC recommends initiation of antiretroviral therapy within 36 hours of a needle-stick injury from a potential HIV source. Studies show that as time elapses after exposure, viral replications exceed the control of the antiretroviral therapy. After 72 hours, therapy to prevent infection is no longer effective.[20,31] Combination therapy should consist of two or more antiretroviral drugs and continue for 4 weeks. Laboratory evaluation for adverse effects should be considered after 2 weeks. Health care workers with questions about post-exposure prophylaxis (PEP) should contact the CDC PEPline at 1 (888) HIV-4911 (1-888-448-4911).

Recent HIV research studies have demonstrated a cross-reacting antibody that develops late in HIV infection of patients with decreased CD4+ cells and high viral loads, which has a neutralizing effect on HIV proteins. The antibody is specifically targeted at the envelope protein, gp120. This antibody response, although too late to eliminate HIV infection once established, may prove to be effective in finding a means for HIV immunization.[32]

★ **TABLE 22-7** Common Laboratory Findings in HIV Infections

- Leukopenia
- Anemia
- Macrocytosis (in patients treated with zidovudine)
- Thrombocytopenia
- Decreased CD4 counts
- Positive molecular tests for HIV-1 RNA

☑ CHECKPOINT 22-3

Why does infection with HIV result in an increased chance for opportunistic infections?

Other Acquired Immune Deficiency Disorders

Some disorders are characterized or accompanied by functional abnormalities of lymphocytes. The lymphocyte count can be normal but in many cases is decreased. Acquired defects of either T or B lymphocytes can result in serious clinical manifestations. Some inflammatory states transiently impede the response of T lymphocytes to antigen. These include idiopathic granulomatous disorders and malignancy. Severe infection by one microorganism sometimes impedes the ability of T lymphocytes to react to other infectious organisms. Starvation or severe protein deficiency also can severely affect the functional ability of the T lymphocyte.

Congenital Immune Deficiency Disorders

Congenital disorders are usually characterized by a decrease in lymphocytes and impairment in either cell-mediated immunity (T lymphocytes), humoral immunity (B lymphocytes), or both (Table 22-8 ★). In contrast to the reactive morphologic heterogeneity of lymphocytes associated with viral disorders, lymphocytes in congenital disorders are usually normal in appearance. The functional impairment of the immune response is often apparent from birth or a very young age when children have repeated infections. With continued research and more sensitive techniques, the genetic mutations causing immunodeficiency are being identified.

Severe Combined Immunodeficiency Syndrome

Severe combined immunodeficiency (SCID) syndrome includes a heterogeneous group of disorders resulting in major qualitative immune defects involving both humoral and cellular immune functions. These disorders include numerous and diverse genetic mutations with different inheritance patterns and varied severity in clinical manifestation. Most are inherited as sex-linked or autosomal-recessive traits, but some autosomal dominant forms have been reported. About 75% of individuals with SCID are males because the most common form of SCID is an X-linked disorder.

Both the T and B lymphoid lineages are functionally deficient. Subgroups of SCID are now defined on the basis of which lymphocytes

★ **TABLE 22-8** Laboratory Findings in Selected Immunodeficiency Disorders

Disorder	Immunoglobulins	B Lymphs	T/NK Lymphs	Genetic Loci
SCID				
X-linked				
$T^- B^+ NK^-$	↓ IgG, IgE, IgA; N IgM	N; abnormal function	Absent	*IL2RG* (Xq13.1)
Autosomal recessive				
$T^- B^+ NK^+$	↓ IgG, IgE, IgA; N IgM	N; abnormal function	T lymphs absent	*ZAP-70* (2q12); *IL7R* (5p13)
$T^- B^- NK^+$	↓ IgG, IgM, IgA, IgE	Absent	T lymphs absent	*RAG-1* and RAG-2 (11p13); PNP (14q13.1)
$T^- B^+ NK^-$	↓ IgG, IgE, IgA; N IgM	N; abnormal function	Absent	*JAK3* (19p13.1)
$T^- B^- NK^-$	↓ IgG, IgM, IgA, IgE	Absent	Absent	*ADA* (20q13.11, ADA deficiency); *AK2* (1p34, reticular dysgenesis)
MHC class II deficiency $T^{+/-} B^+ NK^+$	↓ IgG, IgE, IgA; N IgM	N	N or ↓ T lymphs	*CIITA* (16p13); *RFXANK* (19p12); *RFX5* (1q21); *RFXAP* (13q14)
Other Immunodeficiency Syndromes				
X-linked				
X-linked agammaglobulinemia ($T^+ B^- NK^+$)	↓ IgG, IgM, IgA	Mature cells absent	N	*BTK* mutations (Xq21.3-q22)
Wiskott-Aldrich syndrome ($T^+ B^- NK^+$)	↓ IgM; N/↑ IgA; ↑ IgE; N/↑ IgG	N; abnormal function	N or ↓ T lymphs	*WAS* mutations (Xp11.22-11.3)
Autosomal				
diGeorge syndrome ($T^- B^+ NK^+$); AD	N or ↑ IgG; IgM; IgA	N number; delayed maturation	T lymphs absent	*TBX1* (del 22q11.2)
Ataxia-telangiectasia; AR	N/↑ IgM; N/↑ IgG; IgE; IgA	N; abnormal function	↓ T lymphs	*ATM* (11q22-23)
Hyper IgE syndrome (HIES); AR and AD	N IgM, IgG, IgA; ↑ IgE	N	↓ T lymphs	*DOCK8* (9p24.3); *STAT3* (17q21.31)

↓ = decreased; ↑ = increased; N = normal; ADA = adenosine deaminase; AK2 = adenylate kinase 2; AD = autosomal dominant; AR = autosomal recessive; ATM = ataxia telangiectasia mutated; BTK = Bruton's tyrosine kinase; CIITA = Class II MHC transactivator; DOCK8 = dedicator of cytokinesis 8; IL2RG = interleukin 2 receptor, gamma chain; IL7R = interleukin 7 receptor 7 alpha chain; JAK3 = Janus kinase 3; PNP = purine nucleoside phosphorylase; RAG = recombination activating gene; RfX5 = Regulatory factor X-5; RfXAP = RfX associated protein; RfXANK = RfX-associated ankyrin containing protein; SCID = severe combined immunodeficiency syndrome; STAT3 = signal transducer activator of transcription 3; TBX1 = T-box-containing transcription factor; WAS = Wiskott-Aldrich syndrome; ZAP70 = ζ-chain associated protein kinase-70

are absent. If only the T cells are absent, it is termed $T^- B^+ NK^+$ *SCID*; if both T and B cells are absent, it is $T^- B^- NK^+$ *SCID*; and in those patients (usually with the most severe lymphopenia) in whom all three lymphocyte subsets are absent, it is $T^- B^- NK^-$ *SCID*.[33] In all cases both the T and B lymphoid lineages are functionally deficient. Because T-cell help is required in generating an antibody response against protein antigens, disorders in which T lymphocytes are absent result in peripheral blood B lymphocytes that are unresponsive to most mitogens, and immunoglobulin production is decreased. However, these B lymphocytes respond normally when incubated with normal T lymphocytes in vitro.[34] The absolute lymphocyte count is variable but often is decreased to $<1.0 \times 10^9$/L. Lymphocyte counts in SCID patients may be normal in the neonate, especially if they are able to generate B cells and NK cells, but eventually they develop a profound lymphopenia.

Lymph node examination reveals a lack of plasma cells, B lymphocytes, and T lymphocytes. No lymphoid cells are found in the spleen, tonsils, or intestinal tract. The bone marrow also is deficient in plasma cells and lymphocyte precursors.

Frequent recurrent infections, skin rashes, diarrhea, and failure to thrive are characteristic findings in infants with SCID. Death related to overwhelming sepsis usually occurs within the first 2 years of life if untreated. Bone marrow transplantation, immunoglobulin therapy, and gene therapy are the only options for successful treatment.[34]

Sex-Linked SCID. Classic sex-linked (X-linked) SCID is the most common form of inherited severe combined immunodeficiency, accounting for ~45% of cases. This form of SCID ($T^- B^+ NK^-$ SCID) has been mapped to the long arm of the X chromosome (Xq13.1–13.3) and is associated with a loss-of-function mutation in the gene for the γ-chain of the IL-2 receptor (Table 22-8). Because the γ-chain also is an essential subunit of the receptors for IL-4, IL-7, IL-9, IL-15, and IL-21, hematopoietic regulation by all of these cytokines is impaired (Chapter 4). IL-7/IL-7R signaling is required for T-cell development, and in its absence, T-cell lymphopoiesis fails. Thus, X-linked SCID is characterized by absent T lymphocytes, NK lymphocytes, and a hypoplastic thymus.[34] B lymphocytes are normal in number but are nonfunctional due to lack of T-helper function, and immunoglobulin levels are severely depressed. Family history is important in determining the mode of inheritance of SCID, although a negative family history of the disease does not rule out an X-linked disease. Up to one-third of the cases present as a spontaneous mutation.[34]

Females who carry the abnormal X-linked SCID gene have normal immunity. These carriers can be detected by molecular assays for IL-2R mutations in the γ-chain locus using cells other than lymphocytes. The normal mature female cell population is a mosaic with one or the other X chromosome inactivated. In female SCID carriers, however, only lymphocytes carrying normal non-inactivated X chromosomes are found, rather than the expected mixture of cells with normal and abnormal X chromosome inactivation. It has been shown that random X chromosome inactivation occurs in these carriers, but the gene product of the mutant X chromosome does not support lymphocyte maturation. Thus, lymphocytes with the mutant X chromosome fail to develop, and the only lymphocytes found in carriers have the active normal X chromosome.

☑ CHECKPOINT 22-4

Would you expect female carriers of X-linked SCIDS to be more susceptible to infection than the normal population? Why or why not?

Autosomal SCID. The autosomal forms of SCID exhibit severe deficiencies of both T and B lymphocytes. The most common form, found in about 30–40% of autosomal-recessive SCID (15% of all SCID patients), is due to an adenosine deaminase (ADA) deficiency that results in a depletion of T, B, and NK lymphocytes.[35,36] The adenosine deaminase gene is located at chromosome 20q13.11 (Table 22-8). Both point mutations and gene deletions have been associated with ADA deficiency. Another enzyme deficiency, purine nucleoside phosphorylase (PNP), is the cause of ~2% of SCID cases. The gene for PNP is located at chromosome 14q13.1. Both of these enzymes degrade purines. Without PNP, accumulation of toxic DNA metabolites (deoxyadenosine triphosphate/dATP and deoxyguanosine triphosphate/dGTP) occurs, inhibiting normal T- and B-cell development. The two disorders can be differentiated by the presence of NK cells and the ADA enzyme in PNP.[33] Other defects include a deficiency of MHC class II gene expression, interleukin-2 receptor α-chain (IL-2Rα) deficiency, mutations in the *RAG-1* and *RAG-2* genes (which catalyze VDJ recombination), and defective assembly of the T-cell receptor–CD3 complex (Chapter 8).[33]

CASE STUDY *(continued from page 415)*

All of Heidi's immunoglobulin levels were decreased. T and B lymphocyte counts were severely decreased. The peripheral blood smear showed anisocytosis, poikilocytosis (schistocytes), polychromatophilia, and two nucleated RBCs/100 WBCs. Her thymus was not detectable on chest films.

5. Is this child more likely to have a congenital or acquired immune deficiency?
6. If she has a congenital immune deficiency, is it more likely that she has X-linked or autosomal SCIDS?
7. Are the lymphocytes more likely to be morphologically heterogeneous or homogeneous? Why?
8. What confirmatory test is indicated?

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is a sex-linked recessive disease characterized by the triad of eczema, thrombocytopenia, and immunodeficiency resulting in recurrent infections. About two-thirds of affected children have a family history of the disease; in one-third there is an apparent spontaneous mutation. Untreated, most children die before 10 years of age as a result of infection or bleeding. Those who survive longer can develop neoplasms of the histiocytic, lymphocytic, or myelocytic lineages. EBV infections in WAS patients can lead to lymphoma.[37]

★ **TABLE 22-9** Laboratory Features in Wiskott-Aldrich Syndrome (WAS)

Feature	Description
Platelets	Decreased concentration, small size, abnormal platelet function
Lymphocytes	Decreased or normal concentration, T lymphocytes variable, B lymphocytes usually normal
Immunoglobulin	IgM decreased, IgE and IgA increased, IgG normal/increased
Antibodies to blood group antigens	Absent or decreased
PCR	Detects WAS gene mutation

Ig = immunoglobulin; PCR = polymerase chain reaction

Laboratory findings play an important role in the diagnosis of WAS (Table 22-9 ★). There is a progressive decrease in thymic-dependent immunity and depletion of paracortical areas in the lymph nodes, leading to abnormal lymphocyte function. The absolute numbers of CD4+ and CD8+ T lymphocytes and their ratio is normal to variable. Typically, CD8+ T lymphocytes are decreased, but numbers vary over time for individuals. Circulating B lymphocyte numbers are normal, but antibody production is abnormal.[37] Serum IgM levels are decreased, but IgE and IgA levels are increased. IgG concentrations are usually normal (Table 22-8).

One of the most consistent findings is the low or absent level of circulating antibodies to the blood group antigens.[37] These children are unable to produce antibodies to polysaccharide antigens, a T lymphocyte-independent phenomenon. This suggests that there is an intrinsic B lymphocyte abnormality.

A low platelet count with abnormal bleeding in the neonatal period is one of the first clinical signs of WAS. Patients have a severe thrombocytopenia ($<70 \times 10^9$/L) with small-size platelets.[37] Platelets are intrinsically abnormal with decreased expression of surface glycoproteins IIb, IIIa, and IV and the defective expression of CD62P and CD63. Bleeding times are abnormal, but the prothrombin time and activated partial thromboplastin time are normal, indicating the coagulation factor proteins are adequate (Chapters 31 and 32). Megakaryocytes in the bone marrow are normal or increased in number and morphologically normal. Genetic mutations involve the WAS gene on the short arm of the X chromosome between Xp11.22 and Xp11.3. Females are carriers; affected males do not pass the deficiency to their male children. PCR techniques detect 98% of affected males and are the primary diagnostic tests when WAS is suspected.[37] Molecular analysis using restriction fragment length polymorphisms reveals that female carriers have selective inactivation of the WAS X chromosome rather than random inactivation of paternal or maternal X chromosomes and are asymptomatic. This nonrandom inactivation pattern is found in the carrier's T and B lymphocytes, granulocytes, monocytes, and megakaryocytes, indicating that all hematopoietic cells in WAS are affected by the presence of the *WAS* gene.

Therapy includes treatment for bleeding and infection. Splenectomy usually results in correction of the platelet count and normalized platelet volume and significantly reduces the risk of bleeding complications. Bone marrow transplant before 5 years of age has an 85% cure rate.[37]

☑ CHECKPOINT 22-5

What laboratory findings suggest WAS in a child, and how is the diagnosis confirmed?

DiGeorge Syndrome

DiGeorge syndrome is a congenital immunodeficiency marked by the absence or hypoplasia of the thymus, hypoparathyroidism, heart defects, and dysmorphic facies. Hypocalcemia is typical, and the presenting symptom can be seizure resulting from hypocalcemia. Usually a decrease in peripheral blood T lymphocytes occurs as well as a decrease in the cellularity of the T lymphocyte regions of peripheral lymphoid tissue. The low lymphocyte count is related to a decreased number of CD4+ lymphocytes (Table 22-8). T lymphocyte function varies. Children with a hypoplastic thymus may be able to produce enough lymphocytes with normal function to maintain immunocompetence. B lymphocytes are normal in number and function, and immunoglobulin levels are normal. Infants exhibit increased susceptibility to viral, fungal, and bacterial infections that are frequently overwhelming. Death occurs in the first year unless thymic grafts are performed.

Cytogenetic studies on these children show a chromosome 22q11.2 deletion.[38] This defect also is found in a parent of a child with DiGeorge syndrome in 25% of the cases.

Sex-Linked Agammaglobulinemia

Sex-linked (X-linked) agammaglobulinemia (Bruton's disease) is inherited as a sex-linked disease characterized by frequent respiratory and skin infections with extracellular, catalase-negative, pyogenic bacteria. Molecular analysis has revealed that the genetic defect is on the long arm of the X chromosome, (Xq21.3–22; Table 22-8).[34] More than 90% of patients have a loss-of-function mutation of a tyrosine kinase gene, Bruton's tyrosine kinase (*BTK*). The genetic mutation results in a block in B lymphocyte maturation at the pre-B lymphocyte stage. The variable and constant regions of the IgM immunoglobin chain fail to connect. Peripheral blood lymphocyte counts are normal as are T lymphocytes; there is, however, a decrease in B lymphocytes and an absence of plasma cells in lymph nodes. The serum concentrations of IgG, IgM, and IgA are decreased or absent. Cell-mediated immune function is normal. Monthly injections of gamma globulin are effective in preventing severe infections.

Female carriers of this disease have normal immunity. All of their B lymphocytes carry the paternal, normal X chromosome, suggesting that the normal X chromosome confers a survival advantage.

Ataxia-Telangiectasia

Ataxia-telangiectasia (AT) is inherited as an autosomal-recessive disease that results from mutations in the ataxia-telangiectasia mutated (*ATM*) gene at chromosome 11q22–23. The ATM protein is involved in signaling pathways involved in cellular responses to DNA damage. The disease is characterized by progressive neurologic disease, immune dysfunction, and predisposition to malignancy. Affected individuals are ataxic and in childhood or adolescence develop telangiectasias. A telangiectasia is a vascular lesion formed by a dilation of a group of blood vessels that appears as a red line or radiating limbs (spider). Chronic respiratory infection and lymphoid malignancy are the most common causes of death.

Patients with AT have a defect in cell-mediated immunity with hypoplasia or dysplasia of the thymus gland and depletion of T lymphocyte areas in the lymph nodes. B lymphocyte function also is abnormal with impaired isotype switching (Chapter 8). Lymphocytopenia with a reversed CD4:CD8 ratio exists along with deficiencies in IgA, IgG, and IgE. IgM levels are increased. Cytogenetic analysis reveals excessive chromosome breakage and rearrangements in cultured cells and clonal abnormalities of chromosome 7 or 14.[39]

Hyper IgE Syndrome (HIES)

Advanced molecular techniques in high-resolution genomic hybridization have identified genetic mutations in previously unclassified or unknown SCIDS. Several mutations have recently been linked to immune deficiencies resulting from decreased cell signaling, decreased lymphocyte counts, and decreased immunoglobulins except for an elevated IgE. These **hyper IgE syndromes (HIES)** are associated with several mutations (Table 22-8). The dedicator of cytokinesis 8 (DOCK8) mutation shows an autosomal recessive inheritance pattern.[40] This mutation impairs T lymphocyte function, which results in a lack of specific antibody formation, recurrent viral infections, candidiasis, dermatitis, and the increased serum IgE.[40]

JAK-STAT (Chapter 4) mutations alter cytoplasmic signaling pathways, which cause deficiencies in multiple cell lineages. CD4+ lymphocytes, B lymphocytes, myeloid cells, keratinocytes, osteoclasts, and monocytes all are decreased. A recently found mutation in the signal transducer and activator of transcription 3 (STAT3) protein shows an autosomal-dominant inheritance pattern and is the most frequent cause of immune deficiency with HIES. [41] STAT3 is necessary for normal IL-17 cytokine signaling and regulation. Deficiencies in IL-17 due to STAT3 mutations lead to decreased differentiation of T-helper 17 (Th17) lymphocytes[41] and result in a primary immunodeficiency. Both T lymphocytes and NK lymphocytes are decreased. IL-17 also controls eosinophilotropic chemokines that drive eosinophil production. Patients have an eosinophilia, numerous tissue abnormalities including facial, dental, skeletal and soft tissue abnormalities, short limbs, and osteoporosis and are susceptible to repeated infections.[41] Recurrent infections are characterized by increased levels of serum IgM but deficiencies in the other immunoglobulins, especially IgG.

Both X-linked and autosomal recessive mutations have been identified that affect T lymphocyte activation because of abnormalities in cytokine signaling. Patients have qualitative defects in both T and B lymphocytes with recurrent infections and extreme susceptibility to opportunistic infections.[33]

Summary

Lymphocytes mount an immune response in inflammatory or infectious states. In these states, the lymphocyte morphology often includes various reactive forms, immunoblasts, and possibly plasmacytoid cells. Quantitative changes (either increased or decreased) in the total lymphocyte concentration occur. Although the lymphocyte induces an immune response to eliminate foreign antigens, the cell itself also can serve as the site of infection for some viruses that use lymphocyte membrane receptors to attach to and invade the cell.

Infectious mononucleosis is a common self-limiting lymphoproliferative disorder caused by infection with EBV. Laboratory diagnosis of this disorder includes serologic testing for heterophile antibodies and identification of reactive lymphocytes on Romanowsky-stained blood smears.

AIDS is a disease caused by infection of the CD4+ lymphocyte with the retrovirus HIV-1. The virus suppresses the immune response by replicating within and destroying CD4+ lymphocytes. CD4+ lymphocyte levels and viral loads monitor the disease's progression. Antiretroviral treatments in combination with protease inhibitors slow the progression of the disease. It currently has no cure, but research is investigating a neutralizing immune response that may lead to a vaccine.

Congenital qualitative disorders of lymphocytes include a wide variety of immunodeficiency disorders. Either the T or B lymphocyte or both can be affected. These are usually very serious defects with most affected individuals succumbing to the disease in childhood. As molecular testing improves detection, more genetic mutations will elucidate the origins of these primary immunodeficiencies so that treatment options can be explored. Bone marrow/stem cell transplant is the only treatment in many cases, but enzyme therapy and gene modification are being evaluated.

Review Questions

Level I

1. Advanced HIV disease (AHD) patients have numerous infections because the HIV virus has infected: (Objective 9)

   A. neutrophils

   B. CD8+ lymphocytes

   C. CD4+ lymphocytes

   D. B lymphocytes

2. According to the CDC definition, a patient is considered to have progressed from AHD to AIDS when the following is detected: (Objective 10)

   A. a leukopenia

   B. a CD4:CD8 ratio of 2:1

   C. the absolute CD4+ count of <200/mcL

   D. the absolute CD4+ count of <500/mcL

3. Which of the following infectious agents is associated with a newborn found to have jaundice and microcephaly with an enlarged spleen and decreased platelet and red cell counts? (Objective 7)
   A. cytomegalovirus
   B. Epstein-Barr virus
   C. *Toxoplasmosis gondii*
   D. *Bordetella pertussis*
4. A teenager is seen at the clinic for a sore throat and enlarged cervical lymph nodes. He has reactive, atypical lymphocytes along with an elevated CRP and elevated aminotransferases. He should also be tested for: (Objectives 1, 3)
   A. pertussis antitoxin antibodies
   B. HIV antibodies
   C. Toxoplasmosis antibodies
   D. heterophile antibodies
5. A 29-year-old female patient was seen for abdominal fullness, fever, and lethargy. Her total leukocyte count was 11.8×10^9/L. The differential showed 32% neutrophils, 65% lymphocytes, and 3% monocytes. A few of her lymphocytes were small round lymphocytes with a high N:C ratio and overall size similar to the red cells, but many of her lymphocytes appeared large with basophilic cytoplasm and increased N:C ratio. This patient should be considered for: (Objective 5)
   A. a urinary tract infection
   B. hepatitis
   C. bacterial meningitis
   D. *Streptococcus pneumonia*
6. The morphology of lymphocytes found in infectious mononucleosis is described as: (Objective 2)
   A. segmented nucleus with pinkish-tan cytoplasm
   B. horseshoe-shaped nucleus with blue/gray cytoplasm
   C. irregular shaped nucleus with spreading, deep blue cytoplasm
   D. small cells with condensed chromatin and deep blue cytoplasm
7. A 2-year-old child has a total leukocyte count of 10×10^9/L and 60% lymphocytes. Which of the following best describes the child's blood count? (Objectives 4, 8)
   A. absolute lymphocytosis
   B. relative lymphocytosis
   C. normal lymphocyte count for the age given
   D. absolute lymphocytopenia

Use this case study for questions 8–10.

A 19-year-old female college student went to student health complaining of lethargy and a sore throat for the past two weeks. Physical exam shows pharyngitis, lymphadenopathy, and splenomegaly with a total leukocyte count of 11×10^9/L and 70% lymphocytes (50% of lymphs are reactive).

8. She probably has: (Objective 1)
   A. HIV
   B. hepatitis
   C. X-linked SCIDS
   D. infectious mononucleosis
9. Her absolute lymphocyte count is: (Objective 4)
   A. 10×10^9/L
   B. 11×10^9/L
   C. 5.5×10^9/L
   D. 7.7×10^9/L
10. The best description of this patient's leukocyte count is a(n): (Objectives 4, 8)
    A. relative lymphocytopenia
    B. relative neutrophilia
    C. absolute lymphocytosis
    D. absolute neutrophilia

Level II

1. A 17-year-old female patient is seen at the clinic because she has a lingering cough. She had a total leukocytosis of 23×10^9/L with 79% normal-looking lymphocytes. She should be screened for: (Objective 6)
   A. pertussis antitoxin antibodies
   B. VCA IgM
   C. EBNA
   D. CMV antibodies
2. A patient with lethargy, pharyngitis, and lymphadenopathy is found to have many lymphocytes that are large with spreading, irregular cytoplasm and some with deep basophilia. A study of the lymphyocyte subsets would be expected to show: (Objective 2)
   A. decreased reactive B lymphocytes with increased cytotoxic T lymphocytes
   B. increased reactive B lymphocytes with decreased cytotoxic T lymphocytes
   C. decreased reactive B lymphocytes with decreased cytotoxic T lymphocytes
   D. increased reactive B lymphocytes with increased cytotoxic T lymphocytes

3. Epstein-Barr virus infects lymphocytes by attaching to which receptor? (Objective 2)

   A. CD4

   B. CD8

   C. CD21

   D. TCR

Use this case study to answer questions 4 and 5.

A young male sees his physician for an ongoing cough. He is found to have a total leukocyte count of 28 $\times$ 10^9/L with a differential of 22% neutrophils, 72% lymphocytes, 5% monocytes, and 1% eosinophils. The majority of the lymphocytes appear small with dense chromatin.

4. Which of the following describes his blood count? (Objective 7)

   A. absolute neutrophilia

   B. relative neutrophilia

   C. absolute lymphocytosis

   D. relative eosinophilia

5. The most likely cause of illness is: (Objectives 5, 7, 10)

   A. Epstein-Barr virus

   B. *Bordetella pertussis*

   C. Cytomegalovirus

   D. hepatitis

6. After taking care of a pair of cats, a 25-year old male saw his physician for fever of unknown origin. He had a total leukocyte count of 16 $\times$ 10^9/L with 78% lymphocytes, many showing reactivity, basophilia, and increased cytoplasm. His monospot test was negative. What other testing should be considered? (Objective 4)

   A. screening for *Toxoplasmosis gondii*

   B. screening for HIV

   C. detection of pertussis antitoxin antibodies

   D. detection of hepatitis B antigen

Use this case study for questions 7–9.

A 39-year-old male went to the clinic with complaints of nagging cough, weight loss, diarrhea, and low-grade temperature. Results of physical examination showed lymphadenopathy, congested lungs, and increased heart rate. Slight splenomegaly and hepatomegaly were noted. A CBC and flow cytometry studies were ordered. Histologic examination of sputum with Gomori's methenamine silver nitrate stain revealed *Pneumocystis carinii*.

Laboratory data		**Differential**	
WBC count	2.8 $\times$ 10^9/L	Segmented neutrophils	68%
RBC count	3.86 $\times$ 10^{12}/L	Lymphocytes	21%
Hb	13.6 g/dL (136 g/L)	Monocytes	10%
Hct	0.41 L/L	Eosinophils	1%
Platelet count	104 $\times$ 10^9/L	Positive for HIV-1 antibodies	

7. What clinical condition does this patient have? (Objectives 8, 10)

   A. congenital immune deficiency

   B. infectious mononucleosis

   C. AHD

   D. AIDS

8. Which lymphocytes are periodically counted to monitor the disease? (Objective 8)

   A. infected B lymphocytes

   B. CD4+ T lymphocytes

   C. CD8+ T lymphocytes

   D. natural killer lymphocytes

9. Which laboratory test will be used to follow this patient's disease? (Objective 8)

   A. HIV-1 viral load

   B. throat swab

   C. serologic test for heterophile antibody

   D. PCR for genetic mutations

10. Immune deficiency associated with HIES is attributed to the abnormal signaling or loss of cytokine: (Objective 11)

    A. IL-2

    B. IL-7

    C. IL-15

    D. IL-17

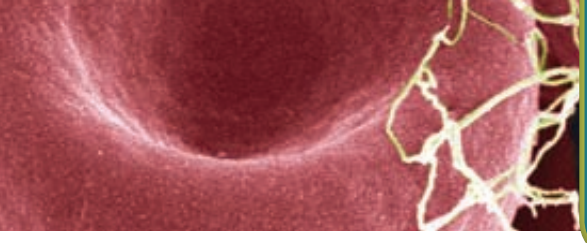
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Straus SE, Cohen JI, Tosato G et al. Epstein-Barr virus infections: biology, pathogenesis, and management. *Ann Intern Med*. 1993;118:45–58.
2. Foerster J. Infectious mononucleosis. In: Lee GR, Foerster J, Lukens JN et al., eds. *Wintrobe's Clinical Hematology*, 10th ed. Baltimore: Williams & Wilkins; 1999:1926–55.
3. Bailey RE. Diagnosis and treatment of infectious mononucleosis. *Am Fam Physician*. 1994;49:879–85.
4. Huang SC, Chen JS, Cheng CN, Yang YJ. Hypoalbuminaemia is an independent predictor for hemophagocytic lymphohistiocytosis in childhood Epstein-Barr virus-associated infectious mononucleosis. *Eur J Haematol*. 2012;89:417–22.
5. Paul JR, Bunnell WW. The presence of heterophile antibodies in infectious mononucleosis. *Am J Med Sci*. 1932;183:90–104.
6. Ebell M. Epstein-Barr virus infectious mononucleosis. *Am Fam Physician*. 2004;70:1279–87.
7. Vouloumanou EK, Rafailidis PI, Falagas ME. Current diagnosis and management of infectious mononucleosis. *Curr Opin Hematol*. 2012;19:14–20.
8. Taylor M, Lennon B, Holland C et al. Community study of toxoplasma antibodies in urban and rural school children age 4 to 8 years. *Arch Dis Child*. 1997;77:406–9.
9. Dropului L. The impact of cytomegalovirus infections on solid organ transplantation. *Adv Stud Med*. 2006;6:303–4, 319–28.
10. Steininger C. Clinical relevance of cytomegalovirus infection in patients with disorders of the immune system. *Clin Microbiol Infect*. 2007;13(10):953–63.
11. Mustafa MM. Cytomegalovirus infection and disease in the immunocompromised host. *Pediatr Infect Dis J*. 1994;13:249–57.
12. Arnez M, Cizman M, Jazbec J, Kotnik A . Acute infectious lymphocytosis caused by coxsackievirus B2. *Pediatr Infect Dis J*. 1996;15:1127–28.
13. Crowcroft N, Pebody R. Recent developments in pertussis. *Lancet*. 2006;367:926–36.
14. Hodge G, Hodge S, Markus C et al. A marked decreased in L-selectin expression by leucocytes in infants with *Bordetella pertussis* infection: leucocytosis explained? *Respirology*. 2003;8:157–62.
15. Faulkner A, Skott T, Martin S et al. Pertussis. *VPD Surveillance Manual*. 2011. Chapter 10. www.cdc.gov/vaccines/pubs/surv-manual/chpt10-pertussis; accessed March 4, 2012.
16. Dworkin MS. Adults are whooping, but are internists listening? *Ann Intern Med*. 2005;142:832–35.
17. Troussard S, Cornet E, Lesesve JF et al. Polycolonal B-cell lymphocytosis with binucleated lymphocytes (PPBL). *Onco Targets Ther*. 2008;1:59–66.
18. Schoentag RA, Cangiarella J. The nuances of lymphocytopenia. *Clin Lab Med*. 1993;13:923–36.
19. Gottlieb MS, Schroff R, Schanker HM et al. *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med*. 1981;305:1425–31.
20. Mofenson LM, Brady MT, Danner SP et al. Guidelines for the prevention and treatment of opportunistic infections among HIV-exposed and HIV-infected children: recommendations from CDC, the National Institutes of Health, the HIV Medicine Association of the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the American Academy of Pediatrics. *MMWR Recomm Rep*. 2009;58(RR-11):1–166.
21. Gold JWM. HIV-1 Infection. *Med Clin North Am*. 1992;76:1–18.
22. Centers for Disease Control and Prevention (CDC). Revised surveillance case definition for HIV infection–United States, 2014. *MMWR Recomm Rep*. 2014;63(RR-03):1–10.
23. Liebman HA, Tulpule A. Hematologic manifestations of AIDS. In: Hoffman R, Furie B, Benz EJ Jr et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone; 2013:2191–207.
24. Said JW. Pathogenesis of HIV infection. In: Nash G, Said JW, eds. *Pathology of AIDS and HIV Infection*. Philadelphia: WB Saunders Co.; 1992;26:15–18.
25. Brynes RK, Gill PS. Clinical characteristics, immunologic abnormalities, and hematopathology of HIV infection. In: Joshi VV, ed. *Pathology of AIDS and Other Manifestations of HIV Infection*. New York: Igaku-Shoin; 1990:21–42.
26. Doukas MA. Human immunodeficiency virus associated anemia. *Med Clin North Am*. 1992;76:699–709.
27. Aboulafia DM, Mitsuyasu RT. Hematologic abnormalities in AIDS. *Hematol Oncol Clin North Am*. 1991;5:195–214.
28. Hammer SM, Eron JJ, Reiss P et al. Antiretroviral treatment of adult HIV infection. *JAMA*. 2008;300(5):555–70.
29. Hutter G, Nowak D, Mossner M et al. Long-term control of HIV by *CCR5* Delta 32/Delta 32 stem cell transplantation. *N Engl J Med*. 2009;360:692–98.
30. Allers K, Hütter G, Hofmann J et al. Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. *Blood*. 2011;117:2791–799.
31. New York State Department of Health AIDS Institute. HIV prophylaxis following occupational exposure. *NYSDoH AI:*Albany; 2010:2–10. www.hivguidelines.org; accessed Feb. 28, 2012.
32. Grays ES, Madiga MC, Hemanus T et al. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T-cell decline and high viral load during acute infection. *J Virol*. 2011;85:4828–40.
33. Lewis DB, Nadeau KC, Cohen AC. Disorders of lymphocyte function. In: Hoffman R, Furie B, Benz EJ et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Churchill Livingstone; 2009:721–45.
34. Davis J, Puck JM. X-linked severe combined immunodeficiency. *GeneReviews*. 2005. www.ncbi.nlm.nih.gov/Pubmed/20301584; accessed March 4, 2012.
35. Hersfield M. Adenosine deaminase deficiency. *GeneReviews*. 2011. www.ncbi.nlm.nih.gov/Pubmed/20301656; accessed February 25, 2012.
36. Rosen FS, Bhan AK. Weekly clinicopathological exercises, case 18-1998: a 54-day-old premature girl with respiratory distress and persistent pulmonary infiltrates. *N Eng J Med*. 1998;338:1752–58.
37. Filipovich AH, Johnson J, Zhang K. WAS-related disorders. *GeneReviews*. 2011. www.ncbi.nlm.nih.gov/Pubmed/20301357; accessed March 4, 2012.
38. Fomin AB, Pastorino AC, Kim CA et al. DiGeorge syndrome: a not so rare disease. *Clinics (Sao Paulo)*. 2010;65:865–69.
39. Forte WC, Menezes MC, Dionigi PC et al. Different clinical and laboratory evolutions in ataxia-telangiectasia syndrome: report of four cases. *Allergol Immunopathol*. 2005;33:199–203.
40. Su HD, Jing H, Zhang Q. Dock8 deficiency. *Ann NY Acad Sci*. 2011;1246:26–33.
41. Tangye SG, Cook MC, Fulcher DA. Brief reviews: insights into the role of STAT3 in human lymphocyte differentiation as revealed by the hyper-IgE syndrome. *J Immunol*. 2009;182:21–28.



23 Introduction to Hematopoietic Neoplasms

SHIRLYN B. McKENZIE, PhD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define and differentiate the terms *neoplasm* and *malignant* and identify hematopoietic disorders that can be included in each category.
2. Compare and contrast the general characteristics of the myelodysplastic syndromes (MDSs), myeloproliferative neoplasms (MPNs), and acute and chronic leukemias.
3. Describe the World Health Organization (WHO) classification system used for MDSs, MPNs, the leukemias, and lymphoid neoplasms.
4. List the various laboratory methods used to classify the hematopoietic neoplasms.
5. Compare and contrast the laboratory findings of the acute and chronic leukemias and myeloid and lymphoid leukemias.
6. Differentiate proto-oncogenes and oncogenes and summarize their relationship to neoplastic processes.
7. Correlate patient age to the overall incidence of the hematopoietic neoplasms.
8. Explain the usefulness of immunological techniques, chromosome analysis, molecular genetic analysis, and cytochemistry in the diagnosis and prognosis of hematopoietic neoplasms.
9. State the prognosis and survival rates of the hematopoietic neoplasms.
10. Define and differentiate proto-oncogenes and oncogenes.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Explain how proto-oncogenes are activated and the role that oncogenes and tumor suppressor genes and their protein products play in the etiology of hematopoietic neoplasms.
2. Describe the effects of radiation on the incidence of leukemia.
3. Differentiate between the acute and chronic myeloid and lymphoid leukemias based on their clinical and hematologic findings.
4. Reconcile the use of chemotherapy for treatment of leukemia.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

5. Compare and contrast treatment options for the hematopoietic neoplasms including possible complications.
6. Name the leukemogenic factors of leukemia and propose how each contributes to the development of leukemia.
7. Compare and contrast laboratory features of MDS, MPN, acute myeloid and lymphoid leukemia (AML, ALL), and mature lymphoid neoplasms, and justify a patient diagnosis based on these features.
8. Define the principles of, explain the applications of, and select appropriate cytochemical stains for bone marrow evaluation of hematopoietic neoplasms.
9. Select laboratory procedures appropriate for confirming cell lineage and diagnosis in hematopoietic neoplasms.
10. Define *epigenetics* and explain its role in cancer.
11. Define *cancer stem cell* and explain its similarities to the hematopoietic stem cell (HSC).

Key Terms

Acute lymphocytic leukemia (ALL)
Acute myeloid leukemia (AML)
Auer rod
Benign
Cancer-initiating cell
Cancer stem cell
Chronic lymphocytic leukemia (CLL)
Chronic myelogenous leukemia (CML)
Consolidation therapy
Cytogenetic remission
Epigenetics
French-American-British (FAB)
Hematologic remission
Induction therapy
Leukocyte alkaline phosphatase (LAP)
Leukemia
Leukemic hiatus
Leukemic stem cell
Leukemogenesis
Lymphoma
Maintenance chemotherapy
Malignant
Mature neoplasm
Minimum residual disease (MRD)
Molecular remission
Myeloperoxidase (MPO)
Myelodysplastic syndrome (MDS)
Myeloproliferative neoplasm (MPN)
Neoplasm
Nonspecific esterase
Oncogenes
Periodic acid Schiff (PAS)
Precursor neoplasm
Proto-oncogene
Specific esterase
Tartrate resistant acid phosphatase (TRAP)
Tumor suppressor gene

Background Basics

The information in this chapter serves as a general introduction to the hematopoietic neoplasms (Chapters 24–28). To maximize your learning experience, you should review the following concepts before starting this unit of study:

Level I

- Summarize the origin and differentiation of the hematopoietic cells. (Chapters 2, 4)
- Describe the morphologic characteristics of the hematopoietic cells. (Chapters 5, 7, 8)

Level II

- Describe the actions of cytokines, cytokine receptors, signaling pathways, and transcription factors. (Chapters 2, 4)
- Summarize the cell cycle and identify factors that affect it. (Chapter 2)
- Describe the normal structure and function of the bone marrow, spleen, and lymph nodes. (Chapter 3)

CASE STUDY

We will refer to this case study throughout the chapter.

Agnes, a 72-year-old female, saw her physician for a persistent cough and fatigue. She had always been in good health and played golf regularly. Upon examination, she was noted to be pale and had slight splenomegaly. A CBC revealed the WBC count was 83.9 $\times$ 10^9/L.

Consider possible explanations for this test result and which reflex tests should be performed.

OVERVIEW

This chapter is the first in a section that discusses the hematopoietic neoplasms. It provides a general introduction to neoplastic hematologic disorders that are discussed in detail in Chapters 24–28. It describes oncogenesis including how oncogenes are activated and their association with hematopoietic disease. This is followed by a description of how neoplasms are classified and characterized according to cell lineage, degree of cell differentiation, morphology, cytochemistry, immunophenotype, and genetic abnormalities. The etiology and pathophysiology of the leukemias are examined using general clinical and laboratory findings. Finally, prognosis and treatment modalities for the disorders are considered.

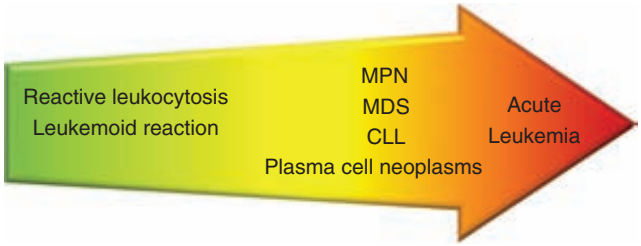
A comprehensive table that includes the diagnosis, immunophenotype, chromosome translocation, and genotypic finding is included in Appendix B. This table should be referred to when reading the chapters in this section. This introductory chapter should be read before progressing to the other chapters in this section. The other chapters are organized according to the World Health Organization (WHO) classification of hematopoietic and lymphopoietic neoplasms. Chapter 24 includes the myeloproliferative neoplasms. Chapter 25 includes the myelodysplastic syndromes. The acute myeloid neoplasms are described in Chapter 26, the acute lymphoid neoplasms are described in Chapter 27, and Chapter 28 includes a discussion of the mature lymphoid neoplasms. The chapters on flow cytometry (Chapter 40), chromosome analysis (Chapter 41), and molecular analysis (Chapter 42), are referenced frequently and will provide additional information on laboratory procedures used in diagnosis, prognosis, and treatment.

INTRODUCTION

Neoplasm (tumor) means "new growth." Neoplasms arise as a consequence of dysregulated proliferation of a single transformed cell. Genetic mutations in the transformed cell reduce or eliminate the cell's dependence on external cytokines (growth factors) to regulate proliferation.

Neoplasms are either malignant or benign. **Benign** neoplasms are formed from highly organized, differentiated cells and do not spread or invade surrounding tissue. *Malignancy* means "deadly" or "having the potential for producing death." A **malignant** neoplasm is a clone of abnormal, anaplastic, proliferating cells, which often have the potential to metastasize (spread). Only malignant tumors are correctly referred to as cancer. Although cancer is actually a malignancy of epithelial tissue, common use of the term includes all malignant neoplasms. A benign neoplasm can be premalignant and progress with further genetic mutations to a malignant neoplasm (Figure 23-1 ■).

Malignant neoplasms of the bone marrow are collectively known as **leukemia**. These neoplasms are grouped according to cell lineage as lymphoid, myeloid, and histiocytic/dendritic cell.[1] The myeloid and lymphoid neoplasms are further subgrouped as **precursor** (acute) and **mature** (usually chronic) **neoplasms**. A comparison of clinical and laboratory findings associated with these precursor and mature subgroups is listed in Table 23-1 ★.



■ **FIGURE 23-1** The spectrum of hematopoietic proliferation ranges from benign to malignant outcomes. Benign myeloid and lymphoid proliferation is usually a reactive process (reactive leukocytosis; leukemoid reaction). Mature myeloid and lymphoid neoplasms include myeloproliferative neoplasms (MPNs), and myelodysplastic syndromes (MDSs), chronic lymphocytic leukemia (CLL), and plasma cell neoplasms. These neoplasms are derived from a mutated precursor cell that divides incessantly but has some capacity to mature. Acute leukemia (AL) is a malignant precursor neoplasm characterized by unregulated cell proliferation and a block in maturation. Mature neoplasms can progress with additional genetic mutations and terminate in AL.

Precursor neoplasms are characterized by genetic mutations that promote proliferation and survival and/or that block differentiation into mature hematopoietic cells. Thus, in acute leukemia (AL), a precursor neoplasm, there is a gap in the normal maturation pyramid of cells with many blasts (Figure 23-2 ■) and some mature forms but a decrease in intermediate maturational stages. This is referred to as the **leukemic hiatus**. The mature cells seen in the bone marrow and peripheral blood arise from proliferation of the residual normal hematopoietic stem cells (HSCs) in the bone marrow. The excess of blasts primarily reflects proliferation of the abnormal malignant clone that fails to undergo maturation.

An increase in proliferation and survival of the neoplastic cells characterizes mature neoplasms, but in contrast to precursor neoplasms, maturation of progenitor cells is nearly normal. The result is leukocytosis with the predominant leukemic cells being amitotic, mature or partially mature cells with normal function. Thus, the bone

★ **TABLE 23-1** Comparison of Precursor and Mature Hematopoietic Neoplasms

	Precursor (acute neoplasm)	Mature (chronic neoplasm)
Age	All ages	Adults
Clinical onset	Sudden	Insidious
Course of disease (untreated)	Weeks–months	Months–years
Predominant cell	Blasts, some mature forms	Mature forms
Anemia	Mild–severe	Mild
Thrombocytopenia	Mild–severe	Mild
WBC	Variable	Increased

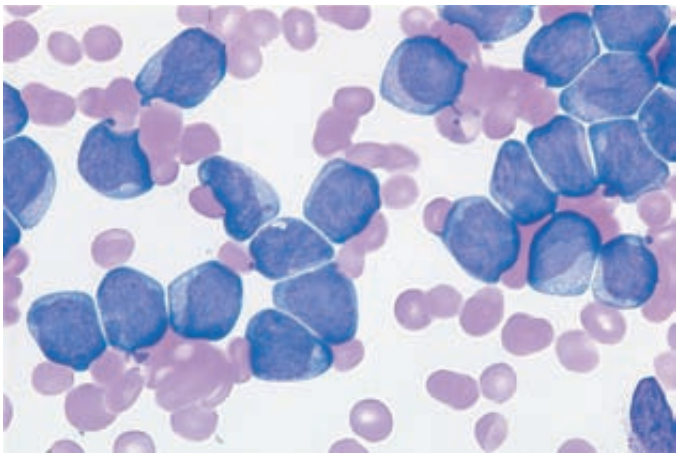


FIGURE 23-2 Acute myeloid leukemia. Note the large number of myeloblasts with no mature granulocytes present (Wright stain, 1000× magnification, peripheral blood).

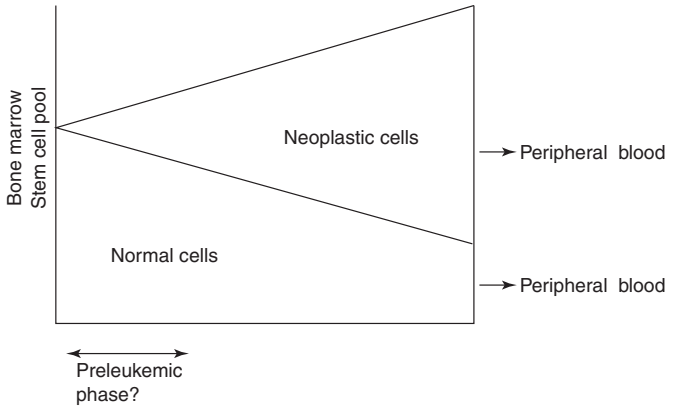


FIGURE 23-4 Clonal expansion of neoplastic cells in the bone marrow over a period of time leads to a decrease in the concentration of normal cells in both the bone marrow and peripheral blood.

marrow and peripheral blood usually show an increase in concentration of cells at all maturation stages (Figure 23-3 ■). The mature myeloid neoplasms include subgroups in which the cells exhibit dysplastic features (myelodysplasia).

Abnormal proliferation of lymphoid cells sometimes occurs within the lymphatic tissue or lymph nodes. These solid tumors are referred to as **lymphoma**. If the lymphoma affects the bone marrow and lymphoma cells are found in the peripheral circulation, the leukemic phase of lymphoma is present.

Failure of normal hematopoiesis is the most serious consequence of malignant neoplasms. As the neoplastic cell population increases, the concentration of normal cells decreases, resulting in the inevitable cytopenias of normal blood cells (Figure 23-4 ■). If the neoplasm is not treated, the patient usually succumbs to infections secondary to granulocytopenia or bleeding secondary to thrombocytopenia.

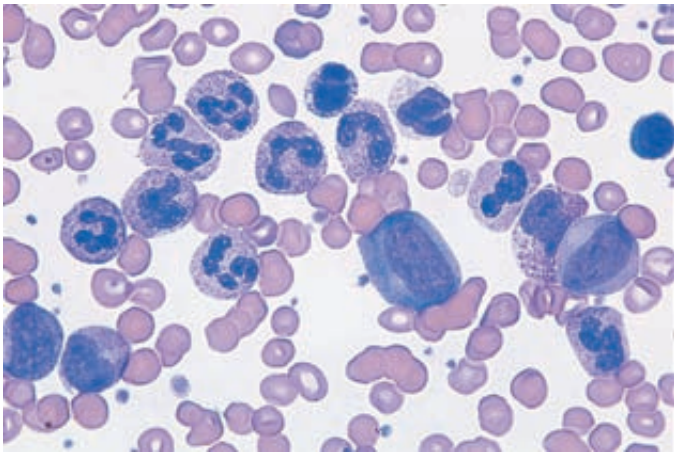


FIGURE 23-3 Chronic myelogenous leukemia. Note the large number of granulocytic cells in various stages of maturation including blasts, metamyelocyte, bands, and segmented neutrophils (Wright stain, 1000× magnification, peripheral blood).

ETIOLOGY/PATHOPHYSIOLOGY

Cancer is a disease of gene mutations. Hematopoietic neoplasms are believed to occur as the result of a somatic mutation(s) of a single hematopoietic stem or progenitor cell.[2] Evidence for the clonal evolution of neoplastic cells comes from cytogenetic studies. More than 50% of individuals with leukemia show an acquired abnormal karyotype in hematopoietic cells whereas other somatic cells are normal. Using cytogenetic markers, normal and malignant cells can be demonstrated to populate the marrow simultaneously. In untreated leukemias and during relapse, the leukemic cells dominate, whereas during remission, usually only normal cells can be detected.

Cancer Stem Cells

Hematopoietic neoplasms arise when a normal hematopoietic precursor cell acquires a cancer-initiating mutation. The cell in which this genetic mutation(s) occurs is termed the *cell of origin* or **cancer-initiating cell** (Figure 23-5 ■). The cancer-initiating cell can be a hematopoietic stem cell (HSC) or more differentiated progenitor cells.[2,3] A hallmark of hematologic malignancies is the capacity for unlimited self-renewal of the cancer-initiating cell, which is also a characteristic of normal HSCs (Chapter 3). While more restricted progenitor cells, such as committed lymphoid progenitor (CLP) or committed myeloid progenitor (CMP) cells, do not *normally* have self-renewal capacity, they may acquire mutation(s) that reactivate the self-renewal program(s) and that allow them to become cancer-initiating cells.[2] Many of the signaling pathways that have been shown to regulate normal stem cell development also play a role in cancer cell proliferation.[2,3]

The cancer-initiating cell gives rise to the **cancer stem cell** that divides incessantly to generate a tumor of "identical" sibling cells or clones and sustains malignant growth. Most cancers are not truly clonal but consist of heterogeneous cell populations. Only small subsets of cells that are capable of extensive proliferation (cancer stem cells) exist within a tumor. Identification of the hematopoietic form of a cancer stem cell, or **leukemic stem cell (LSC)**, for each type of

leukemia has become a major focus of research because the LSC is responsible for propagating the leukemia.

☑ CHECKPOINT 23-1

A patient has 50% monoblasts in the bone marrow. Which precursor cell could be the cancer-initiating cell?

The mutations leading to malignant transformation of the cancer stem cell or LSC often are associated with a chromosome alteration that is observed as an abnormal karyotype when studying cells in mitosis(Chapter 41). When chromosome studies are normal, aberrations in DNA at the molecular level may be found (Chapter 42). In either case, genomic changes in the cancer cells lead to a survival and/or proliferation advantage over normal cells and to the neoplastic expansion of the affected cancer stem cell and its progeny. In acute leukemia, this unregulated proliferation is accompanied by an arrest in maturation at the blast cell stage (Figure 23-5).

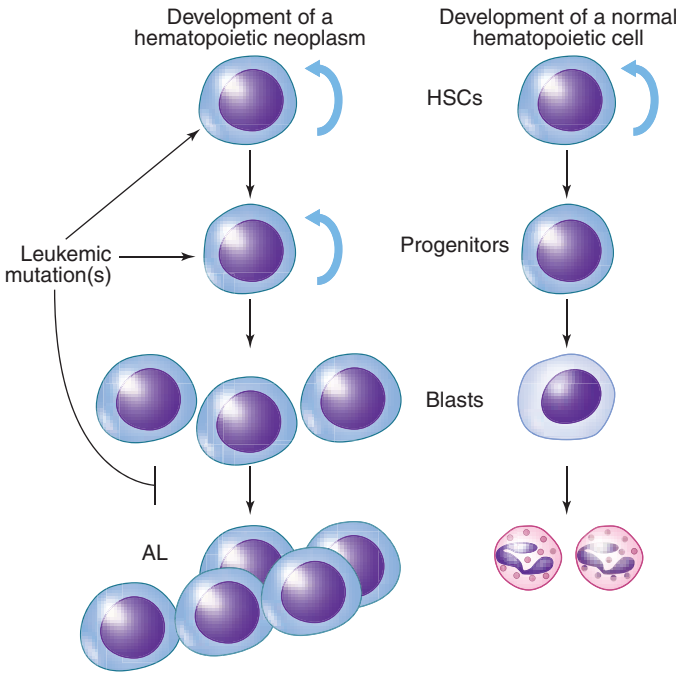


FIGURE 23-5 A hematopoietic neoplasm (left) is derived from a single cancer-initiating cell. The leukemic mutation(s) (black arrows) that transform a normal hematopoietic precursor cell to a cancer-initiating cell can occur at the hematopoietic stem cell (HSC) or more committed progenitor. If the cancer-initiating cell is a committed progenitor, then the mutations must include the capacity for self-renewal (blue arrows). If the mutation also can result in a block to terminal differentiation (⊣) the resulting malignancy will be an acute leukemia (AL). Residual "normal" HSCs and committed progenitors in the marrow will still be capable of producing mature cells (right).

☑ CHECKPOINT 23-2

A 62-year-old male presents with an elevated leukocyte count, mild anemia, and a slightly decreased platelet count. His physician suspects leukemia. Explain why the erythrocytes and platelets are affected.

Molecular Basis of Cancer

The cancer cell genotype is generally maintained (stably inherited) during cell division. This implies that the tumor cell DNA determines the disease phenotype.

Oncogenes

Researchers have found that when inoculated into animals, certain viruses are capable of causing tumors. It has been shown that these tumor viruses carry discrete genetic elements, **oncogenes**, that are responsible for inducing malignant cell transformation. The proteins encoded by the oncogenes play important roles in the cell cycle, such as initiation of DNA replication and transcriptional control of genes. Importantly, many viral oncogenes have normal counterparts in the human genome, now called **proto-oncogenes**. The identification of proto-oncogenes verified that the human genome carries genes with the potential to dramatically alter cell growth and to cause malignancy when altered or activated to an oncogene.[4]

One of the defining features of cancer cells is their ability to proliferate under conditions in which normal cells do not.[5] The proteins encoded by proto-oncogenes function in the signaling pathways by which cells receive and execute growth instructions. The mutations that convert proto-oncogenes to oncogenes (referred to as *oncogene activation*) are often either structural mutations resulting in the continuous (constitutive) activity of a protein without an incoming signal or mutations in gene regulation that lead to the production of a protein at the wrong place or time. The result in either case is a persistent internal growth signal that is uncoupled from environmental controls. It is possible that any gene playing a key role in cellular growth can become an oncogene if mutated in an appropriate way.

In general, the proto-oncogenes that have been identified serve one of the following functions in normal growth control[4] (Chapters 2, 4):

- **Growth factors** These molecules provide the signals to grow and when activated to an "oncogene" result in an autocrine growth stimulation.
- **Growth factor receptors** When activated to an oncogene, the mutated receptors are capable of triggering growth-promoting signals, even in the absence of ligand (cytokine) binding.
- **Signal transducers** The normal function of these proteins (the largest class of proto-oncogenes) is to pass receptor signals

to downstream targets. Many of these proto-oncogenes encode protein-tyrosine kinases found on the inner surface of the membrane. Often the oncogenic form of these genes produces signaling molecules that exist in a constantly activated state in the absence of growth factor/receptor interaction and signaling.

- **Transcription factors** These proteins bind DNA and function to control the expression of cellular genes required for proliferation.

Thus, proto-oncogenes are genes that regulate the initiation of DNA replication, cell division, the commitment to cellular differentiation, and/or apoptosis (Chapter 2). Their activation to an oncogene disrupts the growth-control apparatus of the cell. Proto-oncogene activation occurs by one of three genetic mechanisms: mutation, gene rearrangement, or gene amplification. The result is either (1) a *qualitative* change in function of the gene's protein product, resulting in enhanced activity, (2) a protein that is no longer subject to the control of regulatory factors, or (3) a *quantitative* change (increased production) of an otherwise normal protein.

Tumor Suppressor Genes

Cancer is now widely accepted to be a multi-hit phenomenon, resulting from several independent genetic alterations occurring sequentially within a single cell. Specific **tumor suppressor genes** function to inhibit cell growth in normal cells. Thus, in addition to oncogene activation that results in growth-promoting activity, tumor cells often have inactivating mutations of growth-suppressing genes that can also contribute to tumor development. Mutations in tumor suppressor genes behave differently from oncogene mutations (Table 23-2 ★). Oncogene mutations tend to be activating mutations, which functionally are dominant to wild-type (nonmutated) gene products; they produce proliferation signals even when a single copy of the oncogene is present. Tumor suppressor mutations, on the other hand, are recessive, loss-of-function mutations. Mutation in one gene copy usually has no effect as long as a reasonable amount of normally functioning wild-type protein remains. However, mutations in both copies of a tumor suppressor gene lead to complete loss of its normal, growth-inhibitory effect.

Understanding the function of tumor suppressor genes has been greatly aided by studies of rare cancers that run in families in which affected family members appear to inherit susceptibility to and develop certain kinds of tumors at rates much higher than the normal population. The first of these familial cancers to be explained at the molecular level was the inherited susceptibility to retinoblastoma (a tumor of the eye) in certain families.[6,7] Although retinoblastoma can occur sporadically, about one-third of the cases occur in related siblings, suggesting an inherited susceptibility to the disease (Web Figure 23-1). The development of retinoblastoma requires two mutations that inactivate both of the *RB* loci on each of the chromosomes 13. In the familial form of the disease, the affected children inherit one mutant *RB* allele and one normal allele. Retinoblastoma (or another malignancy) develops when acquired mutations eliminate the function of the remaining normal (wild-type) allele. Thus, the *RB* gene acts as a tumor suppressor gene that normally functions to arrest excessive growth of cells. As is typically true of tumor suppressor genes, one *RB* copy is sufficient to keep growth in check, but loss of both copies eliminates the tumor suppressor function, and a tumor develops. The protein product of the *RB* gene (Rb protein) is not specific to retinal tissue but serves as a universal cell cycle brake in most cells. Acquired mutations of *RB* (i.e., nonfamilial) are found in about 25% of sporadic cancers and have been observed in various hematopoietic neoplasms.

Inactivation of the *p53* gene, also a tumor suppressor gene, is seen in more than half of all human cancers, making it the most common genetic defect detectable in human tumors.[8,9] Interestingly, a damaged *p53* gene can be inherited (like familial retinoblastoma) resulting in Li-Fraumeni syndrome and an inherited susceptibility to a variety of cancers including hematopoietic neoplasms.[10,11] In affected individuals, 50% develop cancer by age 30 and 90% by age 70. The function of p53 in cell cycle regulation (Chapter 2) is to block cell cycle progression in the event of damaged DNA or to trigger apoptosis if the damaged DNA cannot be repaired. The p53 protein is a major component of the body's antitumor army, serving as a "molecular policeman" monitoring the integrity of the genome. Loss of function of the *p53* gene facilitates tumor formation by allowing damaged cells to proceed through the cell cycle and continue to replicate.

Epigenetics

In addition to mutations of various oncogenes or tumor suppressor genes known to be associated with malignancy, there is a second group of alterations, *epigenetic alterations*.[12,13] **Epigenetics** is defined as heritable changes in gene expression not caused by changes in DNA sequence. Epigenetic changes are stable from one cell generation to the next after each mitotic event. These changes play an important role in normal development and differentiation and are associated with

★ **TABLE 23-2** Properties of Oncogenes and Tumor Suppressor Genes

Property	Oncogenes	Tumor Suppressor Gene
Nature of mutation	Dominant (one mutated allele displays the phenotype)	Recessive (both alleles must be mutated to display the phenotype)
	Gain of function	Loss of function
Inherited mutant allele	Never observed	Rare—basis for inherited predisposition in cancers
Somatic mutations in cancers	Yes	Yes

"silencing" genes and chromatin condensation into heterochromatin (Chapter 2). Major epigenetic changes include DNA methylation of CpG dinucleotides and histone acetylation/deacetylation reactions.

Cancer is a complex disorder that often involves aberrant DNA methylation patterns. There can be demethylation of the genome in regions where it should be methylated or methylation of regions of the genome that are typically unmethylated. The list of genes that acquire hypermethylation of CpGs in their promoter regions and contribute to tumorigenesis is growing. This hypermethylation is associated with transcriptional silencing of these genes and is the explanation for one of the most common causes of loss of function of key tumor suppressor genes.

Modifications of the histone proteins also are seen in malignancies. Hypo-acetylated histones bind tightly to the phosphate backbone of DNA and help maintain chromatin in an inactive, silent state. Various types of malignant cells utilize enzymes called *histone deacetylases* (*HDACs*) to deacetylate key lysine amino acids, resulting in the silencing of key genes that favor cell growth over differentiation. One of the newer approaches to treating cancer patients involves *demethylating agents* or *HDAC inhibitors* to reverse epigenetic changes associated with certain types of cancer.[14]

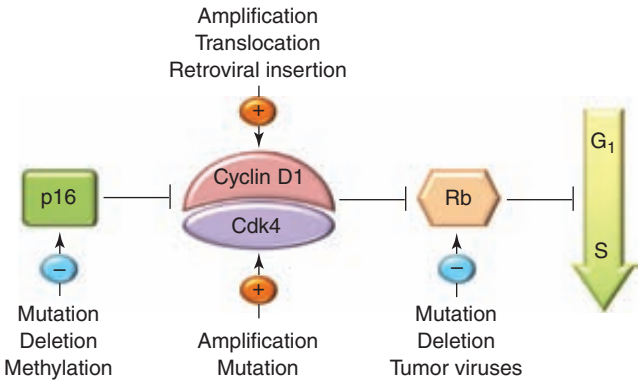


FIGURE 23-6 Alterations of the G_1 checkpoint that can lead to malignancy. Loss-of-function alterations in cell cycle negative regulators (i.e., the tumor suppressor gene products p16 or Rb) can contribute to uncontrolled proliferation. Similarly, gain-of-function mutations of positive regulators of proliferation can contribute to uncontrolled proliferation (i.e., the proto-oncogene gene products Cyclin D, Cdk4).

⊣ = inhibition of the pathway; + = an alteration that increases activity of the indicated proteins; − = an alteration that decreases activity of the indicated proteins

☑ CHECKPOINT 23-3

Mutations in proto-oncogenes predisposing to malignancy are said to be dominant mutations, whereas mutations in tumor suppressor genes are said to behave as recessive mutations, requiring loss of both alleles. Explain this difference in behavior of the gene products.

Cell cycle Checkpoints and Cancer

A common feature of many cancer cells is the loss of regulation of cell cycle checkpoints (Chapter 2), either by overexpression of positive regulators (for example, cyclins and Cdks) or the loss of function of negative regulators (the Cdk inhibitors, p53, or Rb).[15,16] Cyclin D, cyclin E, and cyclin A are overexpressed in a variety of human cancers and function as oncogenes in their mutated configuration. Often specific chromosomal translocations activate the expression of a cyclin gene by placing it under the influence of other transcriptional control elements. For example, the t(11;14) translocation seen in some B-lymphocyte malignancies places the cyclin D gene under regulatory control of the immunoglobulin heavy chain locus, resulting in activation of cyclin D expression at inappropriate times during a cell's life. Thus, the result of the t(11;14) translocation is an oncogenic form of cyclin D (Chapter 27). Furthermore, mutations in cyclin-dependent kinases can also lead to a loss of cell cycle regulation. For instance, mutations that lead to overexpression of Cdk4 (a kinase regulated by cyclin D) have been reported in a number of human tumors and contributes to the excessive growth characteristics of those diseases. Therefore, the p16-cyclin D-Rb pathway, which controls the G_1 checkpoint in cell cycle regulation, is believed to play a pivotal role in tumorigenesis (Figure 23-6 ■). Some investigators have proposed that a mutation involving at least one member of this checkpoint must occur in order for a malignant phenotype to be established.[17]

☑ CHECKPOINT 23-4

A cell contains a mutation that blocks expression of p16. What is the effect (if any) on the daughter cells produced?

Apoptosis and Cancer

The accumulation of an excess number of cells, a characteristic of malignancies, can result from increased cell proliferation (see previous discussion of cell cycle checkpoints and cancer) and/or to decreased cell death (apoptosis).[5] Thus, mutations of genes important in regulating apoptosis have also been identified as oncogenes and tumor suppressor genes. These include loss-of-function mutations that initiate apoptosis such as p53, Bax, and other pro-apoptotic Bcl-2 family members as well as overexpression of antiapoptotic proteins such as Bcl-2 and other Bcl-2 family members that function to inhibit apoptosis[18] (Chapter 2). Bcl-2 is overexpressed in most cases of B-cell follicular lymphoma, many cases of B-cell **chronic lymphocytic leukemia (CLL)**, and some cases of **acute myeloid leukemia (AML)**[19] (Chapters 26-28). Mutations in Bax (resulting in loss of pro-apoptotic function) have been reported in about 20% of leukemic cell lines. The result is production of cells with an extended life span, increased proliferation capacity, and diminished cell death.

Leukemogenesis

From studies on laboratory animals, several factors have been suggested as playing roles in **leukemogenesis**: (1) genetic susceptibility, (2) somatic mutation, (3) viral infection, and (4) immunologic dysfunction (Table 23-3 ★).

★ **TABLE 23-3** Factors that May Play a Role in Leukemogenesis

Factor	Example
Genetic susceptibility	Down syndrome
	Fanconi anemia
	Kleinfelter syndrome
	Bloom syndrome
	Wiskott-Aldrich syndrome
	Diamond-Blackfan syndrome
	Xeroderma pigmentosum
	Li-Fraumeni syndrome
Somatic mutation	Radiation
	Chemicals
	Drugs
Viral infection	Retroviruses—HTLV-I, II, V, HIV-1
Immunologic disorders	Wiskott-Aldrich syndrome
	Bruton type X-linked agammaglobulinemia
	Ataxia telangiectasia
	Immunosuppressive therapy

Genetic Susceptibility

Strong evidence suggests that hereditary factors and abnormal genetic material have important leukemogenic effects. A number of individuals who have congenital abnormalities associated with karyotypic abnormalities have a markedly increased risk of developing acute leukemia. Each of these genetic events has the potential to activate proto-oncogenes or eliminate the function of a tumor suppressor gene. The best known of the genetic abnormalities associated with leukemia is Down syndrome in which the extra chromosome 21 may participate in translocations.[20,21] Various other congenital disorders also are associated with an increased risk for leukemia.[22,23]

Somatic Mutation

A somatic cell mutation is an acquired change in the genetic material of cells other than those involved in reproduction. Mutations in the chromosome near proto-oncogenes likely play a role in neoplasm development. More than 50% of patients with leukemia have acquired abnormal karyotypes and cytogenetic studies have revealed specific, consistent mutations in certain subgroups of hematopoietic neoplasms.[23]

Radiation, some chemicals, and drugs can cause chromosome mutations. Ionizing radiation has long been recognized as capable of inducing leukemia, which is evident from observations of human exposure to radiation from nuclear reactions, therapeutic radiation, and occupational exposure to radiation. An increase in leukemia has been observed after treatment with alkylating agents and other chemotherapeutic drugs used in treatment of many kinds of malignancy. The only chemical that is specifically implicated in causing leukemia other than those used as medications is benzene.

The outcome of chromosome breaks and translocations can lead to the activation of oncogenes and ultimately result in the aberrant expression of the protein product (Appendix B). Furthermore, breaks and translocations *within* gene sequences can produce hybrid (fusion) genes and a *new* protein product. For example, the balanced translocation between chromosomes 9 and 22 in **chronic myelogenous leukemia (CML)** results in the formation of a *BCR-ABL* fusion gene that encodes an abnormal tyrosine kinase (TK) protein. The TK is constitutively activated to transduce signals of cell survival, proliferation, and resistance to apoptosis. Consequently, the cell containing the translocation is able to persist under conditions that would not normally support its survival.

Viral Infection

Retroviruses have been shown to cause leukemia in laboratory animals, and a few malignancies can be traced to a viral infection of cells in humans.[24] Retroviruses contain a reverse transcriptase that allows them to produce a DNA copy of the viral RNA core. The DNA can then be copied to produce more viral cores or can be incorporated into the host cell's nuclear DNA. The strongest support for the existence of a leukemogenic virus in humans comes from the isolation of several human retroviruses known as *human T-cell leukemia/lymphoma virus* (HTLV-I, II, V) and *human immunodeficiency virus* (HIV-1) from cell lines of patients with mature T-cell malignancies.[25] Exactly how viruses induce leukemia is unclear, but the incorporation of the viral genome into host DNA is suspected to lead to activation of proto-oncogenes.

Immunologic Dysfunction

An increased incidence of lymphocytic leukemia has been observed in both congenital and acquired immunologic disorders. These disorders include the hereditary immunologic diseases Wiskott-Aldrich syndrome, Bruton type X-linked agammaglobulinemia, and ataxia telangiectasia. An association between long-term treatment of patients with immunosuppressive drugs (e.g., renal transplant) and leukemia also has been observed. Possibly, a breakdown in the cell-mediated immunologic self-surveillance system and/or deficient production of antibodies against foreign antigens leads to the emergence and survival of neoplastic cells.

Miscellaneous Factors

Certain hematologic diseases appear to pose a leukemogenic risk. Leukemia development in some patients appears to be related to the treatment used for the primary disease, but in others, no such relationship can be found. The highest incidence of acute leukemia is found in individuals with other neoplastic bone marrow disorders, such as **myeloproliferative neoplasms (MPNs)** and **myelodysplastic syndrome (MDS)**,[26–28] prompting some hematologists to use the word *preleukemia* or *premalignant* for these disorders. Additional genetic mutations (or epigenetic alterations; see "Epigenetics" section) occur as the preleukemic disease progresses to the malignancy, leukemia. Other hematopoietic diseases with an increased incidence of leukemia include paroxysmal nocturnal hemoglobinuria (PNH), aplastic anemia, and multiple myeloma. Interestingly, all of these hematologic disorders are considered stem cell disorders in which the primary hematologic defect lies in the myeloid or lymphoid progenitor cells or in the pluripotential stem cells.

No single factor is responsible for the activation of oncogenes that result in hematopoietic neoplasms, but a variety of etiologic

factors including genetic factors and environmental exposures produce the malignancy.[29–31] The cause probably varies from patient to patient, and some individuals could be more susceptible than others to oncogene activation.

☑ CHECKPOINT 23-5

Does a 3-year-old child with Down syndrome have an increased risk of developing leukemia? Why or why not?

EPIDEMIOLOGY

The Leukemia and Lymphoma Society predicted that in 2013, about 150,000 new cases of leukemia, lymphoma, and myeloma would have been reported, accounting for 9% of new cases of cancer in the United States.[32] About 31% more males are living with leukemia than females.

Approximately 50% of all leukemias are diagnosed as acute. Although some difference in the incidence of the acute leukemias exists between countries and regions of countries, the differences are not great. However, all leukemias are more prevalent in Jews of Russian, Polish, and Czech ancestry than in non-Jews. Acute leukemia also is more common in whites than in blacks.

Of particular interest are the incidence and morphologic variation of leukemia among age groups (Table 23-4 ★). Although acute leukemia occurs at all ages, peak incidence occurs in the first decade, particularly from the ages of 2 to 5 followed by a decreasing incidence in the second and third decade. Thereafter, the incidence begins to increase, rising steeply after age 50. The cellular type of leukemia occurring at these peak periods differs significantly. Most childhood acute leukemias are of the lymphoid type, whereas those occurring in adults are typically myeloid. Chronic leukemias are rare in children. Chronic myelogenous leukemia occurs most often in young to middle-aged adults, and chronic lymphocytic leukemia is found primarily in older adults. MPN and MDS occur most often in middle-age to older adults.

CLINICAL FINDINGS

Failure of the normal triad of hematopoiesis is the most serious consequence of hematopoietic neoplasms. The most frequent symptoms are related to erythrocytopenia, thrombocytopenia, and/or neutropenia. The major clinical problems are anemia, infection, and bleeding episodes occurring as hemorrhages, petechiae, or ecchymoses. Bone pain that results from marrow expansion and weight loss is also a common complaint. Physical examination can show hepatosplenomegaly and, occasionally, lymphadenopathy. Organomegaly is more common in mature cell leukemias (chronic leukemias) than in the precursor (acute) forms.

Although the disease originates in the bone marrow, neoplastic cells can infiltrate any tissue of the body, especially the spleen, liver, lymph nodes, central nervous system, and skin. The lesions produced vary from rashes to tumors. Skin infiltration is most commonly found in acute myeloid leukemia (AML), particularly those with a monocytic component. Central nervous system (CNS) involvement is common in **acute lymphoblastic leukemia (ALL)** of childhood.[33] Chloromas, which are green tumor masses of immature leukocytes, are associated with AML and CML and are usually found in bone but can be found throughout the body. The green color, which fades to a dirty yellow after exposure to air, is responsible for the descriptive name given to this unique clinical finding. Presumably, the green color results from the myeloperoxidase content of the malignant cells.

★ **TABLE 23-4** Age Groups Typically Found in Acute (Precursor) and Chronic (Mature) Leukemias

Neoplasm	Age
• Acute lymphocytic leukemia (ALL)	Children 2–5 years old
• Chronic lymphocyte leukemia (CLL)	Adults >50 years old
• Acute myeloid leukemia (AML)	Adults
• Chronic myeloid leukemia (CML) and other myeloproliferative neoplasms	Adults

HEMATOLOGIC FINDINGS

Cell counts and morphology are variable in the hematopoietic neoplasms (Table 23-5 ★). A normocytic (occasionally macrocytic) normochromic anemia is usually present at diagnosis. If not present initially, anemia invariably develops during progression of the disease.

The platelet count varies. Thrombocytopenia is usually present at diagnosis in precursor neoplasms (acute leukemia). Thrombocytosis is a common initial finding in some of the mature neoplasms but can decrease with disease progression. Platelet morphology and function also can be abnormal. Large hypogranular platelet forms are common, and circulating micromegakaryocytes occasionally are present.

The leukocyte count can be normal, increased, or decreased. More than 50% of patients with AML do not have a significant leukocytosis at diagnosis. However, if left untreated, leukocytosis eventually develops. On the other hand, in the mature myeloproliferative and lymphoproliferative neoplasms, leukocytosis at diagnosis is a prominent finding. Normal or decreased leukocyte counts are typical in myelodysplastic syndrome (MDS). Regardless of the leukocyte count, an increase in immature precursors is found in most cases. Blasts are especially prominent in the precursor leukemias. Unique pink-staining granular inclusions called **Auer rods** can be found in the blast cells and promyelocytes of some acute myeloid leukemias. These Auer rods are believed to be formed from fused primary granules. When AML is suspected, finding Auer rods can help to establish the diagnosis because they are not found in ALL.

The bone marrow is hypercellular but occasionally is normocellular or hypocellular. Reticulin is increased, often worsening with disease progression. Blasts are usually increased. A minimum of 20% blasts is recommended for a diagnosis of AML. The cutoff of <20% blasts is used to differentiate the mature myeloid neoplasms (MPN and MDS) from precusor myeloid neoplasms.

Maturation abnormalities are commonly present in all three cell lines. Megaloblastoid erythropoiesis can be prominent but is unresponsive to vitamin B_{12} or folic acid treatment.

★ **TABLE 23-5** Characteristic Hematologic Findings in Hematopoietic Neoplasms

Neoplasm	Leukocytes	Platelets	Bone Marrow	Other
Acute leukemia	Normal or increased; blasts present	Decreased	Hypercellular, >20% blasts	Auer rods in AML
MDS	Normal or decreased; blasts can be present	Variable	Hypercellular, occasionally hypocellular, <20% blasts	Dysplastic
MPN (includes chronic myelogenous leukemia (CML)	Increased; shift to left but predominance of mature forms	Usually increased	Hypercellular, occasionally hypocellular, <20% blasts	
MDS/MPN	Increased blasts and other immature forms	Usually decreased	Hypercellular, <20% blasts	Dysplastic
CLL	Increased mature lymphocytes; neutropenia	Variable	Hypercellular, <20% blasts	Smudge cells; <10% prolymphocytes

MDS = myelodysplastic syndrome; MPN = myeloproliferative neoplasm; CLL = chronic lymphocytic leukemia

Because of the intense increase in cell turnover, other laboratory tests reflecting cell destruction could be abnormal. An increase in uric acid, which is a normal product of nucleic acid metabolism, is a consistent finding in all types of leukemia. The rate of excretion can increase to 50 times normal. Serum lactic dehydrogenase (LD) levels appear to correlate closely with the concentration of leukemic cells. Isoenzyme studies reveal that the LD is derived from immature leukocyte precursors. Muramidase (lysozyme) is a lysosomal enzyme present in monocytes and granulocytes. The serum and urine muramidase concentration in leukemia is highly variable and is related to the cellular type. The highest concentrations are found in neoplasms with a monocytic component.

☑ CHECKPOINT 23-6

Why is finding Auer rods an important factor in the diagnosis of leukemia?

HEMATOPOIETIC NEOPLASM CLASSIFICATION

Classifications of hematopoietic neoplasms are considered to be important for three reasons:

1. They provide clinicians and researchers a way to study, select, and compare various therapeutic regimens.
2. They provide a system for diagnosis using clearly defined clinical features and laboratory findings.
3. They permit meaningful associations of genetic abnormalities with pathogenesis of neoplastic disease.

Before 2001, hematopoietic neoplasm classification was primarily based on cell lineage as determined by the morphology and cytochemistry of the neoplastic cells. The system was known as the **FAB (French-American-British)** classification system.[34] Later, immunophenotyping was added to the schema. More recently, genetic features, prior therapy, and a history of myelodysplasia are recognized to have a significant impact on the clinical behavior of the hematopoietic neoplasms. Thus, in 2001, the Society for Hematopathology and the European Association of Hematopathologists developed the World Health Organization (WHO) classification to better define the hematopoietic neoplasms; this classification was updated in 2008.[35,36] This classification uses morphologic, cytochemistry, and immunophenotype analyses to determine cell lineage and degree of maturation. These analyses also help to determine whether cell proliferation is effective or ineffective and to detect whether cells are cytologically normal or dysplastic. Clinical features such as prior therapy, age, and history of MDS are correlated with genetic findings, morphology, and immunophenotype to define subgroups of myeloid and lymphoid neoplasms.[35,36] Cytogenetic and molecular genetic studies are necessary to categorize those neoplasms that are genetically defined, to establish a baseline to assess genetic evolution and progression, and to monitor response of the disease to therapy. In some cases, molecular analysis can detect gene mutations when the cytogenetic profile is normal. These studies that define cell lineage, degree of differentiation, and dysplasia and detect genetic abnormalities should be performed at initial evaluation.[36]

The classification according to lineage of the neoplastic cells includes three groups: myeloid, lymphoid, and histiocytic/dendritic (Table 23-6 ★). Mast cells are derived from hematopoietic progenitor cells and possess myeloid cell characteristics. Thus, mast cell disease can also be considered a myeloid disorder. These major groups are defined and subgrouped as precursor (acute) and mature (chronic) neoplasms by cell morphology, genetic abnormalities, immunophenotyping, and clinical features. Each neoplasm is proposed to have a normal counterpart. (Appendix C is a comprehensive classification according to the 2008 WHO criteria.)

Myeloid Neoplasms

The precursor myeloid neoplasms include the acute leukemias, myeloid sarcoma, and blastic plamacytoid dendritic cell neoplasms. The acute myeloid leukemias are further subgrouped according to primary involved neoplastic cell line (granulocytic, monocytic, erythroblastic, megakaryocytic), genetic features, presence or absence of myelodysplasia, and previous therapy. Those that do not meet the criteria for inclusion in one of the defined subgroups are classified as acute myeloid leukemia, not otherwise specified (NOS).

★ **TABLE 23-6** 2008 WHO Classification of Hematopoietic, Lymphopoietic, and Histiocytic/Dendritic Neoplasms[a]

Classification	Diseases Included
Hematopoietic	Myeloproliferative neoplasms (MPNs)
	Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*
	Myelodysplastic/Myeloproliferative neoplasms
	Myelodysplastic syndromes (MDSs)
	Acute myeloid leukemia (AML) and related precursor neoplasms
	Acute leukemia of ambiguous lineage
Lymphopoietic	Precursor lymphoid neoplasms (T and B lymphoblastic leukemia/lymphoma)
	Mature B-cell neoplasms (including chronic lymphocytic leukemia, CLL)
	Mature T-cell and NK-cell neoplasms
	Hodgkin lymphoma
Histocytic and Dendritic	

[a] Subgroups not included. See Appendix Table 2 for classification that includes subgroups.

The mature myeloid neoplasms are grouped according to genetic and biologic features (myeloproliferation with effective hematopoiesis and myeloproliferation with ineffective hematopoiesis [dysplasia]). These myeloid neoplasms include myeloproliferative neoplasms (MPNs), myelodysplastic/myeloproliferative neoplasms, myelodysplastic syndromes (MDSs), and myeloid/lymphoid neoplasms with eosinophilia and abnormalities of platelet derived growth factor receptors (PDGFRA or PDGFRB) or fibroblast growth factor receptor (FGFR1) (Table 23-6).

An accurate blast count is essential for diagnosis because the blast count in the blood and bone marrow is used to define and differentiate precursor (acute) from mature myeloid neoplasms. The percentage of blasts is determined as the number of blasts in all nucleated marrow cells (except for acute erythroleukemia).[35] Blast counts should be derived from a 200-cell differential on peripheral blood and on a 500-cell differential on bone marrow.[35] The analysis of CD34+ cells by flow cytometry should not be used to assess the blast count because not all leukemic blasts are CD34+. If precursor myeloid leukemia exists concurrently with another nonmyeloid hematologic neoplasm, the cells from the nonmyeloid neoplasm are not included when performing the differential.[35] Counting blasts for a diagnosis of myeloid neoplasms includes the following in addition to myeloblasts:

- Monoblasts and promonocytes in acute monoblastic/monocytic and acute and chronic myelomonocytic leukemia
- Megakaryoblasts in acute megakaryoblastic leukemia
- Abnormal promyelocytes in acute promyelocytic leukemia
- Erythroblasts only in pure erythroleukemia[35]

Lymphoid Neoplasms

The lymphoid neoplasms are classified using the Revised European-American Lymphoma (REAL) classification system as modified by WHO. Similar to the WHO classification of myeloid neoplasms, the REAL/WHO classification of lymphoid neoplasms incorporates immunophenotype, genetics, morphology, and clinical features. The three groups of lymphoid neoplasms are B cell, T/NK cell, and Hodgkin lymphoma (Table 23-6). As with myeloid neoplasms, the T- and B-cell neoplasms are grouped into precursor (lymphoblastic) and mature neoplasms. The ALLs are in the precursor group and include B-cell and T-cell ALL and lymphoblastic lymphoma. Unlike the guideline for AML, no number of blasts is required for a diagnosis of ALL, but it is recommended that a diagnosis of ALL be avoided if the blast count is less than <20%. Some treatment protocols require a threshold of 25% blasts for a diagnosis of ALL. The mature neoplasms can be aggressive and show rapid progression (e.g., plasma cell leukemia), whereas others are indolent (e.g., CLL). Many of the B- and T- cell neoplasms mimic stages of normal T- and B-cell differentiation, so they can be classified to some degree according to their normal B- or T-cell counterpart. However, some do not clearly correspond to a normal cell counterpart (e.g., hairy cell leukemia), some show lineage heterogeneity, some have features of multiple lineages, and others switch lineages.

Most lymphoid neoplasms can be defined using morphology and immunophenotype. However, because a single antigenic marker is not specific for any one neoplasm, panels of monoclonal antibodies are used to help determine the immune profile of the neoplastic clone. Some B-cell neoplasms and a few T-cell neoplasms are associated with specific genetic abnormalities that are important in understanding the biology of the neoplasm as well as in classifying and diagnosing. Morphology, immunophenotype, and genetics of the neoplastic clone should be interpreted within the context of the patient's clinical history. The clinical behavior of lymphoid neoplasms ranges from low grade to high grade, is variable even within one group, and can change with progression of the disease. For this reason, the WHO classification does not use grade to stratify the lymphoid neoplasms. The morphology, immunophenotype, and genetics also can change as the disease progresses.

Hodgkin lymphomas usually occur in young adults, arise in the lymph nodes, and are characterized by the presence of large mononucleated and multinucleated tumor cells called *Reed-Sternberg cells*. The tumor cells can be surrounded by T lymphocytes that form a rosette. The two types are nodular lymphocyte predominant and classical.

Histiocytic and Dendritic Cell Neoplasms

Histiocytic neoplasms develop from myeloid-derived macrophages and dendritic cell tumors develop from myeloid-derived and stromal-derived dendritic cells. The common bone marrow precursor cell for histiocytes and dendritic cells is the common myeloid progenitor (CMP) cell. Histiocytes are derived from blood monocytes, which migrate through vessel walls to reach their destination in tissue, where they develop into histiocytes. Histiocytic tumors are the rarest tumors affecting lymphoid tissue. Dendritic cells are antigen-presenting cells and are found in various locations during different stages of activation, including mucosal sites, skin, and lymph nodes. The types of dendritic cells include Langerhans cells and plasmacytoid dendritic cells. Follicular dendritic cells are thought to be related to bone marrow stromal progenitor cells. There is no marker that identifies all subsets of dendritic cells, and there is no unique phenotypic marker for dendritic or macrophage histiocytes.

CASE STUDY *(continued from page 426)*

The CBC results on Agnes were:

WBC	83.9×10^9/L	**Differential**	
RBC	3.15×10^{12}/L	Segs	12%
Hb	9.5 g/dL (95 g/L)	Lymphs	88%
Hct	29% (0.29 L/L)		
Platelets	130×10^9/L		

1. Given Agnes's laboratory results, would this most likely be considered an acute or chronic leukemia? Explain.
2. What group of leukemia (cell lineage) is suggested by Agnes's blood cell differential results?
3. What would you expect the blast count in the bone marrow to be?

LABORATORY PROCEDURES FOR DIAGNOSING AND CLASSIFYING NEOPLASMS

Initial evaluation of patients with a hematopoietic neoplasm should include collection of peripheral blood and bone marrow specimens prior to therapy. Bone marrow should include aspirate as well as biopsy specimens. Morphology and blast count are performed on Romanowsky-stained smears. In addition to obtaining a blast count, these cells must be differentiated. Differentiation of leukemic blasts allows classification of acute leukemia into cell lineage (lymphoid or myeloid) and various subtypes (Table 23-7 ★). In the acute leukemias, identification of the cell lineage of leukemic blasts is often difficult when performed by morphology alone unless Auer rods are present. However, the distinction of cell lineage is important for selecting the appropriate therapy. Morphology, immunologic marker analysis (immunophenotype), cytochemical stains, and/or genetic studies can differentiate blasts. Cytogenetic analysis on a bone marrow specimen should be performed, and additional molecular genetic studies should be guided by clinical, laboratory, and morphologic information.

Distinction of the mature neoplasms is not as difficult as in acute leukemias because the maturation of the neoplastic cell in mature neoplasms is not blocked, and the cell matures into recognizable blood cells. Immunophenotyping and cytochemistry are not usually necessary in classification of MDS and MPN, but genetic studies are helpful in subgrouping. All data are correlated into a report that gives the WHO diagnosis.[35,36]

Cytochemical Analysis

In hematology, *cytochemistry* refers to in vitro staining of cells that allows microscopic examination of the cells' chemical composition. Cell morphology is not significantly altered in the staining process. Most cellular cytochemical markers represent organelle-associated enzymes and other proteins. The cells are incubated with substrates that react with specific cellular constituents. If the specific constituent

★ **TABLE 23-7** Comparison of Acute Lymphoblastic Leukemia (ALL) and Acute Myeloid Leukemia (AML)[a]

	ALL	AML
Age	Common in children	Common in adults
Hematologic presentation	Anemia, neutropenia, thrombocytopenia, lymphoblasts, prolymphocytes	Anemia, neutropenia, thrombocytopenia, myeloblasts, promyelocytes
Prominent cell morphology	Small-to-medium lymphoblasts, fine chromatin with scanty to abundant cytoplasm, indistinct nucleoli	Medium-to-large myeloblasts with distinct nucleoli, fine nuclear chromatin, and abundant basophilic cytoplasm, possible Auer rods
Cytochemistry	PAS positive, peroxidase negative, SBB negative, TdT positive, chloroacetate esterase (specific) negative	PAS negative, peroxidase positive, SBB positive, TdT negative or positive, chloroacetate esterase positive
Immunophenotype	B Cell: CD19, CD20, cytoplasmic CD79a and CD22, CD10, CD19 (can also have CD13 and CD33); T Cell: CD1a, CD2, CD3, CD4, CD5, CD7, CD8	CD34, HLA-DR, CD117, CD13, CD33, CD15

PAS = periodic acid Schiff; SBB = Sudan black B; TdT = terminal deoxynucleotidyl transferase

[a] Genetic abnormalities are also used to differentiate ALL and AML.

When all cytochemical stains are negative, the differential diagnosis is usually between ALL and AML with minimal differentiation. TdT is not helpful in differentiating the two because it can be positive in 90% of cases of AML with minimal differentiation. Thus, distinction of AML with minimal differentiation from ALL is not possible by cytochemical stains. Flow cytometric analysis and/or ultrastructual cytochemisty is required in this situation. See Chapter 37 for more information on special stains.

is present in the cell, the constituent's reaction with the substrate is confirmed by the formation of a colored product. The stained cells are examined and evaluated on smears with a light microscope, although electron microscopy occasionally is necessary to identify very weak reactions at the subcellular level. The results of these cellular reactions in normal and disease states are well established. Cytochemistry is particularly helpful in differentiating the lymphoid or myeloid lineage of the blasts in precursor leukemias when morphologic identification on Romanowsky-stained smears is difficult (Table 23-7). Cytochemistry is also helpful in subgrouping AML and related precursor neoplasms. Staining procedures are described in Chapter 37.

It is important to remember that the blast cells seen in leukemias are neoplastic and can therefore differ from normal blasts in both morphology and metabolic activity. Leukemic cells often display nuclear/cytoplasmic asynchrony similar to that of megaloblastic cells. As a result, although the nucleus appears very immature, the cytoplasm of leukemic blasts may contain constituents normally present only in more mature cells or can lack one or more constituents expressed by their normal counterparts. In addition, abnormal accumulation and distribution of cellular metabolites is possible.

The cytochemical staining reactions are either enzymatic or nonenzymatic. The enzymatic group includes stains for **myeloperoxidase (MPO)**, esterases, alkaline, and acid phosphatases. The nonenzymatic stains include **Sudan black B (SBB)** for lipids, **periodic acid-Schiff (PAS)** for glycogen, and toluidine blue O for mucopolysaccharides.

MPO and SBB

The MPO and SBB stains are generally performed first to differentiate myeloid and lymphoid blasts. The myeloblasts are positive, but the lymphoblasts are negative. Additional staining may be necessary to assist in subgrouping the AML and ALL.

Esterases

The esterase stains are generally used to differentiate monoblasts and myeloblasts. Myeloblasts are positive for **specific esterases** (chloroacetate esterase), and monoblasts are positive for **nonspecific esterases** (alpha-naphthyl acetate esterase, ANAE).

Periodic Acid Schiff

The PAS stain is helpful in diagnosing ALL and acute erythroid and megakaryoblastic leukemias. Normal erythroblasts do not stain with PAS. However, PAS activity in erythroblasts found in erythroid leukemia is strongly positive. Leukemic lymphoblasts can demonstrate blocklike or coarse granular PAS activity. This typical positivity of lymphoblasts in ALL can sometimes be seen in subgroups of AML (monocytic, erythroleukemia, megakaryoblastic). The PAS stain reaction is not useful in differentiating other acute leukemias (Chapter 37).

Leukocyte Alkaline Phosphatase (LAP)

Leukocyte alkaline phosphatase (LAP) is an enzyme present within the specific (secondary) granules of maturing neutrophils (from the myelocyte stage onward). The enzyme is not present in eosinophils and basophils. Activated neutrophils contain an increased amount of LAP. Therefore, the LAP score is useful in distinguishing leukemoid reactions/reactive neutrophilia (high LAP) from chronic myelogenous leukemia (low LAP).[37,38]

Acid Phosphatase

A constituent of lysosomes and present in most human cells, acid phosphatase activity exists in most normal leukocytes, but the acid phosphatase activity in T-cell ALL is characteristic, exhibiting focal polarized acid phosphatase activity. Hairy cells in hairy cell leukemia are positive, but the activity is not inhibited by tartrate (**tartrate resistant acid phosphatase [TRAP]**), differentiating hairy cells from other leukocytes whose activity is inhibited by tartrate.[39,40]

Terminal Deoxynucleotidyl Transferase (TdT)

A DNA polymerase found in cell nuclei, terminal deoxynucleotidyl transferase (TdT) is a primitive cell marker that is of value in distinguishing ALL from malignant lymphoma[41,42] and is helpful in identifying leukemic cells in body fluid specimens. TdT is present in 90–95% of ALL (T-cell ALL and precursor B-cell ALL, but not in mature B-cell ALL). TdT staining may not be beneficial in evaluating acute leukemias because up to 20% of AMLs can show TdT positivity, and more than 90% of the minimally differentiated AML subgroup can be TdT positive.

Toluidine Blue

Toluidine blue is specifically positive for basophils and mast cells and is useful in diagnosing mast cell disease and rare cases of AML with basophilic differentiation.[43–45] The granules in basophils and mast cells have a strong affinity for toluidine blue. A negative reaction should not rule out a neoplasm of these lineages because the acid mucopolysaccharides can be scarce or negative in neoplastic disorders.

Reticulin Stain and Masson's Trichrome Stain

These stains are used to evaluate both the presence and extent of fibrosis. However, the presence of fibrosis can be suspected on hematoxylin- and eosin- (H&E) stained sections. The Gomori methenamine silver–staining method is more suitable for detecting reticulin. Masson's trichrome stain is useful for evaluating the degree of collagenous fibrosis. Increased staining can be seen in myelofibrosis and indicates severe and dense fibrosis.

Immunologic Analysis

Immunologic analysis is based on identifying specific membrane antigens (surface markers) characteristically found on a particular cell lineage. Immunologic techniques with monoclonal antibodies are widely used to identify cell membrane antigens on a variety of cells (Chapter 40). The development of a large number of monoclonal antibodies that react with surface antigens on normal and neoplastic cells and the technical advances in flow cytometry have greatly enhanced the ability to define leukemic cell lineage, the stage of cell development, and clonality. By utilizing a panel of monoclonal antibodies, a more complete picture of the cells' lineage can be determined.

Immunophenotyping plays a central role in diagnosing hematopoietic and lymphoid neoplasms. It includes identifying the lineage of blasts in the blast phase of CML, subtyping the myeloid leukemias, and identifying the lineage of those leukemias that lack specific morphologic and cytochemical characteristics such as

minimally differentiated AML from ALL and subtyping the lymphoid neoplasms as T or B cell. Immunologic patterns can help plan molecular analysis because some immunophenotypes are associated with particular genetic mutations. The identification of cell antigens can also help direct treatment because some antigens are targeted for specific therapy (e.g., rituximab, an anti-CD20 drug). If marrow cell suspensions are not available, immunophenotyping can be done using immunohistochemical analysis on bone marrow biopsy specimens. See Chapter 40 for a more thorough discussion of monoclonal antibodies and their use in the identification of neoplastic hematopoietic disorders. Appendix B includes the antigenic patterns in hematologic neoplasms.

Genetic Analysis

Genetic analysis includes cytogenetics to determine the cell karyotype and molecular methods to identify specific gene mutations—both of which can be key to differentiate and define some neoplasms. In some cases, the karyotype will appear normal but mutations can be found using molecular techniques such as PCR and fluorescent in situ hybridization (FISH) (Chapter 42).

Cytogenetics

Advances in cytogenetics have enabled cytogeneticists to identify characteristic nonrandom abnormal karyotypes in the majority of the acute leukemias and in some MPNs and MDSs.[46] Some specific chromosome changes are consistently associated with a particular neoplastic subgroup and thus are helpful in diagnosis. For example, the t(15;17)(q22;q12) is diagnostic of acute promyelocytic leukemia, and the Philadelphia chromosome characterized by t(9;22)(q34;q11.2) confirms a clinical diagnosis of CML. In the lymphoid leukemias, nonrandom chromosome changes are often associated with either the B or T lymphocyte lineage and provide important prognostic as well as diagnostic information. Chromosomal rearrangements and their accompanying molecular abnormalities can identify distinct clinical groups with a predictable clinical course and response to specific therapy. In addition to helping physicians evaluate their patients, cytogenetic studies provide new insights into the pathogenesis of neoplastic diseases.

When cytogenetic abnormalities are present before therapy, their presence or absence can be used to identify remission, relapse, and minimal residual disease after therapy. If the cytogenetic abnormality identified before therapy is still present after therapy, it is evidence that neoplastic cells remain in the bone marrow. In some cases, additional chromosome aberrations can be identified during the course of the disease or after a period of remission. The finding of additional chromosome aberrations is not usually a signal of disease progression. Thus, physicians may order multiple cytogenetic analyses (Chapter 41).

Molecular Analysis

Molecular genetic analysis, the process of using DNA technology to identify genetic defects at the molecular level, is being used increasingly as a diagnostic tool in studying neoplasms. In some cases, the chromosome karyotype is normal, but a genetic mutation can be identified. About 5% of patients with CML do not show the typical Philadelphia chromosome on karyotyping, but the *BCR/ABL1* mutation can be identified by molecular techniques. When present, this helps establish or confirm the diagnosis. The *JAK2* mutation, *JAK2*(V617F), plays a pivotal role in the pathogenesis of many *BCR/ABL1*–negative MPNs. Thus, diagnostic algorithms for MPN now consider the mutational status of *JAK2*.

Molecular analysis also is helpful in providing clues to the pathogenesis of hematopoietic neoplasms. For example, the specific t(15;17) (q22;q12) mutation found in acute promyelocytic leukemia produces an abnormal form of the nuclear hormone receptor, retinoic acid receptor-α (RAR-α). The abnormal receptor is involved in the maturation blockade seen in the neoplastic promyelocytic cells. When patients who have this mutation are treated with retinoic acid derivatives, the cells are induced to differentiate into mature granulocytes.

One of the limitations of molecular genetic analysis is that the specific genetic aberration must be identified first so that probes to detect the gene abnormality can be made. Currently, this technology is helpful in diagnosing acute promyelocytic leukemia, CML, polycythemia vera, essential thrombocythemia, primary myelofibrosis, and T- and B-ALL. Another limitation of molecular techniques is that only a single gene mutation usually can be identified using a given probe so that other gene mutations, if present, are not detected. Thus, it is important for the clinical laboratory scientist to understand the advantages and limitations of each diagnostic procedure and recommend the appropriate combination as well as sequence of testing. Chapter 42 discusses molecular genetic techniques and their application in diagnosis of hematopoietic diseases. Appendix B includes the genetic mutations associated with the subgroups of hematologic neoplasms.

Quantitative molecular methods are used to monitor treatment of CML. Baseline levels of mRNA transcripts of *BCR/ABL1* are obtained before treatment is started and then at intervals (3–6 months) to determine whether patients are responding to treatment.[47,48]

☑ CHECKPOINT 23-7

A patient has 35% blasts in the bone marrow. They do not show any specific morphologic characteristics that will allow them to be classified according to cell lineage. What are the next steps that the clinical laboratory scientist should take with this specimen?

PROGNOSIS AND TREATMENT OF NEOPLASTIC DISORDERS

Studies of the hematopoietic neoplasms that have elucidated the pathogenesis of these disorders have led to new treatment modalities that significantly changed the prognosis for patients.

Prognosis

Before the 1960s, a patient diagnosed with acute leukemia could expect to die within a few months. With new treatment modalities, remission rates for both ALL and AML have improved dramatically. Remission was defined originally as a period of time in which there

were no clinical or hematologic signs of the disease. More recently with the various diagnostic and monitoring modalities available, several levels of remission can be defined. For treatment purposes, complete remission (response) is defined as the total absence of disease according to the test used (e.g., hematologic versus cytogenetic versus molecular). **Hematologic remission** refers to the absence of neoplastic cells in the peripheral blood and bone marrow and the return to normal levels of hematologic parameters. **Cytogenetic remission** refers to the absence of recognized cytogenetic abnormalities associated with a given neoplastic disease. **Molecular remission** refers to the absence of detectable molecular abnormalities using PCR or related molecular technologies.

Sensitive molecular testing such as PCR can detect the presence of less than 1 in 10^6 tumor cells (Chapter 42), whereas the sensitivity of detection of malignant cells using cytogenetics is much lower. Thus, a complete molecular response is highly desirable and the most promising evidence that the neoplasm has been eliminated. A combination of negative "traditional" tests (peripheral blood and bone marrow blast count and cytogenetics) and positive molecular tests (PCR/FISH) is sometimes referred to as a state of **minimum residual disease (MRD)**. The designation "partial response" is used when the relevant laboratory values have a significant decrease without achieving a total absence of disease. A "major response" can include either a complete or partial response.

Therapeutic success rates differ by disease and the patient's condition at diagnosis. Often a complete hematologic or cytogenetic remission is achieved initially only to be followed by a return of the disease (relapse) after a period of time. Most currently used treatment regimens target the actively proliferating cancer cells and might not, in fact, be effective against the leukemic stem cells (LSC). Thus, the relapse seen in some patients following a complete remission is likely because of the reemergence of the disease from a quiescent LSC, which was not eliminated by the treatment regimen utilized.

Survival in acute leukemias varies with age and group—ALL or AML. Approximately 80% of children treated for ALL can be expected to enter a prolonged remission with an indefinite period of survival. The prognosis for ALL in adults is not as good as that for children. Two years is the median survival for adults after remission has been achieved. Only 10–25% of patients typically achieve a 5-year survival. The remission rate for AML is about 55–65%. Approximately 50% of these patients remain in remission for more than 3 years. Patients who receive bone marrow or stem cell transplants, especially younger patients, have a better prognosis. Patients who had a previous MDS or chronic MPN respond poorly to standard chemotherapy.

Survival in the chronic neoplasms is longer. The International Randomized Study of Interferon and STI571 (IRIS) reported that for CML patients receiving imatinib as initial therapy, overall survival at 60 months was 89%.[48] After onset of blast crisis, survival is generally only 1–2 months. Survival in CLL depends on its severity at diagnosis and ranges from 30 to more than 120 months. Patients with other MDS and MPN diseases usually survive without treatment for a year or more and even longer for some subtypes. Prognosis for the lymphomas depends on the cell type and can range from 6 months to 10 years or longer.

CASE STUDY *(continued from page 436)*

4. Would you expect Agnes to survive more than 3 years or succumb fairly quickly after treatment?

Treatment

Chemotherapeutic drug and radiotherapy protocols have been developed by cancer and leukemia groups (CALGs) and are used by cooperative oncology groups (COGs) to access statistically valid data in a highly efficient fashion.

Chemotherapy

Chemotherapy remains the treatment of choice for many leukemias. The goal of this type of therapy is to eradicate all malignant cells within the bone marrow, allowing repopulation by residual normal hematopoietic precursors. The problem with this type of therapy is that the drugs used in treatment are not specific for leukemic cells. Thus, treatment also kills many normal cells. Complications of traditional therapy include bleeding because of decreased platelet counts, infections from suppression of granulocytes, and anemia because of erythrocyte suppression in the marrow. Supplemental support with recombinant growth factors can sometimes be used to mitigate the cytopenias.

Most drugs used to treat leukemia are included in three groups: antimetabolites, alkylating agents, and antibiotics. The antimetabolites are purine or pyrimidine antagonists, which inhibit the synthesis of DNA. These drugs kill cells in cycle, affecting any rapidly dividing cell. In addition to leukemic cells, the antimetabolites also kill cells lining the gut, germinal epithelium of the hair follicles, and normal hematopoietic cells. This leads to complications of gastrointestinal disturbances, loss of hair, and life-threatening cytopenias. The alkylating agents (chemical compounds containing alkyl groups) are not specific for cells in cycle but kill both resting and proliferating cells. These drugs attach to DNA molecules, interfering with DNA synthesis. As a class they are mutagenic and carcinogenic; they fragment and clump chromosomes, inactivate DNA viruses, and inhibit mitosis but not protein function. The side effects of these compounds include myelosuppression, stomatitis, nausea, and vomiting. Antibiotics bind to both DNA and RNA molecules, interfering with cell replication. Toxic effects of this therapy are similar to those of alkylating agents.

Since the 1970s, various drug combinations have been found to be more effective than single drug administration. The drugs commonly used and their modes of action are included in Table 23-8 ★.

Therapy for most leukemias is divided into several phases. The **induction therapy** phase is designed to induce the disease into complete remission (i.e., eradicating the leukemic blast population). Once a complete remission has been achieved, it is often followed by a continuation of treatment, referred to as **maintenance chemotherapy** or **consolidation therapy**. The purpose of maintenance therapy is to eradicate any remaining leukemic cells.

The treatment regimen for AML and ALL is similar, although the combination of antileukemic agents differs. However, the purpose of chemotherapy is the same: to eradicate the leukemic blasts. Central nervous system (CNS) involvement is a common feature of ALL but

★ **TABLE 23-8** Chemotherapeutic Agents Usually Used in Acute Leukemia (AL) Treatment

Drug	Class	Action
Doxorubicin	Anthracycline antibiotic	Inhibits DNA and RNA synthesis
Daunorubicin	Anthracycline antibiotic	Inhibits DNA and RNA synthesis
Idarubicin	Anthracycline antibiotic	Inhibits DNA and RNA synthesis
5-Azacytidine	Pyrimidine antimetabolite	Inhibits DNA and RNA synthesis
6-Thioguanine	Purine antimetabolite	Inhibits purine synthesis
Methotrexate	Folic acid antimetabolite	Inhibits pyrimidine synthesis
6-Mercaptopurine	Purine antimetabolite	Inhibits pyrimidine synthesis
Cytosine arabinoside	Pyrimidine antimetabolite	Inhibits DNA synthesis
Prednisone	Synthetic glucocorticoid	Lyses lymphoblasts
Vincristine	Plant alkaloid	Inhibits RNA synthesis and assembly of mitotic spindles
Asparaginase	*Escherichia coli* enzyme	Depletes endogenous asparagines
Cyclophosphamide	Synthetic alkylating agent	Cross-links DNA strands

not of AML. Therefore, CNS prophylactic treatment (cranial irradiation and/or intrathecal chemotherapy) is part of the therapy regimen for ALL.

Permanent remission in CLL is rare. Treatment is conservative and usually reserved for patients with more aggressive forms of the disease. Treatment for MPN and MDS also is primarily supportive and designed to improve the quality of the patient's life. Drugs designed to reverse epigenetic alterations in MDS recently have been introduced (see "Epigenetic Therapy").

Molecular-Targeted Therapy

As the genetic mysteries of hematologic neoplasms are being resolved, novel therapies that target genetic mutations are being developed to silence the gene's expression or that of the mutated protein or to reactivate silenced genes. The therapies appear to be better tolerated than the traditional chemotherapy regimens. Two targeted therapies are in current use as first-line therapy: imatinib for CML and all-trans retinoic acid (ATRA) for acute promyelocytic leukemia.[49] Although hematopoietic stem cell transplantation had been recommended as first-line therapy for CML because it was the only treatment with the potential for cure, recent studies reveal that survival is superior in patients receiving drug treatment (interferon and/or imatinib).[50] Rituximab, cytolytic monoclonal anti-CD20 antibody, is a treatment directed against cells that have the CD20 protein (primarily B cells). These therapies are discussed in Chapters 24 and 26.

Epigenetic Therapy

With the recognition of the contribution of epigenetic alterations to the neoplastic process, a number of drugs have been developed and are in various stages of clinical trials. Both demethylating agents (e.g., azacitidine) and histone deacetylase inhibitors (HDAC-I) (e.g., valproic acid, phenylbutyrate) are being evaluated with promising early results.[51–53]

Bone Marrow Transplant

Bone marrow transplants have provided hope as a possible cure for hematopoietic disorders; the highest rate of success in transplant patients has occurred with those younger than 40 years of age in a first remission with a closely matched donor. In this procedure, drugs and irradiation are used to induce remission and eradicate any evidence of leukemic cells. Bone marrow from a suitable donor is then transplanted into the patient to supply a source of normal stem cells.

Autologous transplants have been used when a compatible donor cannot be found. This procedure involves removing some of the patient's marrow while the patient is in complete remission. The marrow specimen is then treated in vitro with monoclonal antibodies or 4-hydroperoxycyclophosphamide to remove any residual leukemic cells (purged) and cryopreserved. Chemotherapy and/or radiotherapy are administered to the patient to remove all traces of leukemia, and the treated marrow is given back to the patient.

Autologous bone marrow transplantation has been applied to patients in remission and to those in early relapse. Overall survival appears to be better in those transplanted during the first complete remission. Bone marrow transplantation appears to be successful in many cases; the number of patients who are undergoing this type of therapy is increasing.

Stem Cell Transplants

Peripheral blood as well as bone marrow stem cells can be used to re-establish hematopoiesis in the marrow after intensive chemotherapy or radiotherapy in a process called *stem cell transplantation*. In this procedure, apheresis is used to collect stem cells from the peripheral circulation, usually after they have been mobilized (induced to exit the marrow) by cytokines such as G-CSF. These stem cells can come from either the patient (autologous) or from a suitable donor (allogeneic). People who receive allogeneic stem cells are given drugs to prevent rejection. Production of new blood cells usually becomes established in 10–21 days following infusion of the stem cells. Stem cell transplantation is still a fairly new and complex treatment for leukemia. A more thorough discussion of hematopoietic stem cell transplantation can be found in Chapter 29.

CASE STUDY *(continued from page 439)*

5. Is Agnes a suitable candidate for a bone marrow transplant? Why or why not?

Hematopoietic Growth Factors

Recombinant hematopoietic growth factors are used in supportive care of acute leukemia patients. Erythropoietin was introduced in 1989 and has been used in the treatment of chemotherapy-related anemia.[54] Since 1991, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been available to aid in decreasing the incidence of severe neutropenia and infection in patients receiving myelosuppressive chemotherapy.[54] Interleukin-11, introduced for use in 1997,[55] promotes the maturation of megakaryocytes by stimulating stem cells and megakaryocyte progenitor cells. Research in the area of identifying additional hematopoietic growth-stimulating factors continues in the hope of accelerating hematopoietic recovery in chemotherapy patients.

Complications of Treatment

Treatment for leukemia actually can aggravate the patient's clinical situation. Although uric acid levels are commonly elevated in leukemia from an increase in cell turnover, the concentration of this constituent can increase manyfold during effective therapy because of the release of nucleic acids by lysed cells. Uric acid is a normal end product of nucleic acid degradation and is excreted mainly by the kidney. In excessive amounts, the uric acid precipitates in renal tubules, leading to renal failure (uric acid nephropathy). Lysed cells also can release procoagulants into the vascular system, precipitating disseminated intravascular coagulation. In this case, the resulting decrease in platelets and coagulation factors can lead to hemorrhage. This complication is especially prevalent in acute promyelocytic leukemia. The granules of the promyelocytes contain potent activators of the coagulation factors (Chapter 33).

Chemotherapeutics destroy normal as well as leukemic cells. The cytopenias that develop during aggressive chemotherapy can lead to death from infection, bleeding, or complications of anemia. To prevent these life-threatening episodes, the patient can need supportive treatment including transfusions with blood components and/or cytokines as well as antimicrobial therapy.

CASE STUDY *(continued from page 440)*

6. What types of treatment are available for Agnes?

Summary

A neoplasm is an unregulated production of cells that can be either malignant or benign. The WHO classification of neoplastic disorders of the bone marrow hematopoietic cells groups the disorders according to lineage of the neoplastic cells: myeloid, lymphoid, and histiocytic/dendritic. The myeloid and lymphoid neoplasms are subgrouped as precursor and mature neoplasms. Morphology, genetic abnormalities, immunophenotype, and clinical features define these major groups. The mature myeloid subgroups include myeloproliferative neoplasms, myeloproliferative/myelodysplastic neoplasms, and myelodysplastic syndromes. The precursor myeloid group includes the acute myeloid leukemias. The lymphoid group includes precursor B-cell, mature B-cell, precursor T-cell, and mature T-cell neoplasms. The mature lymphoid group includes CLL. The precursor lymphoid group includes ALL.

Oncogenes are altered cellular proto-oncogenes known to contribute to tumorigenesis. Many proto-oncogene protein products are involved in regulating cell growth and include hematopoietic growth factors as well as their cellular receptors, signaling proteins, and transcription factors. Proto-oncogenes can be mutated to oncogenes by mutagens, viruses, or chromosome breaks and translocations. Oncogenes can cause production of abnormal growth factors, abnormal amounts of growth factors, abnormal growth factor receptors, or other abnormalities in the regulatory mechanisms of cell proliferation and differentiation. Epigenetic alterations also play an important role in silencing tumor suppressor genes in neoplastic cells.

Differentiation and classification of acute leukemias depend on accurately identifying the blast cell population. Because the lineage of blast cells is sometimes difficult to differentiate using only morphologic characteristics, immunologic phenotyping using monoclonal antibodies and cytochemical analysis are employed routinely to help identify blast phenotypes and stage of cell differentiation. Chromosome and molecular genetic analyses are helpful because specific mutations often are associated with specific types of leukemias.

Hematologic findings of hematopoietic neoplasms include anemia, thrombocytopenia (in acute leukemia and MDS), and often leukocytosis. A leukocytic shift to the left is consistently found with a combination of blasts and mature cells in acute leukemia. In the chronic leukemias and other chronic neoplastic stem cell disorders, cells appear more on a continuum from immature to mature. Morphologic abnormalities of neoplastic cells are not unusual. Auer rods can be found in blasts of AML.

Historically, hematopoietic neoplasms have been treated using a combination of cytotoxic drugs (chemotherapy). The goal is to induce remission by eradicating the leukemic cells. Hematopoietic stem cell transplants are being used increasingly to restore the marrow after intense chemotherapy or radiotherapy. New approaches include drugs to reverse epigenetic modifications characteristic of neoplastic cells and other drugs targeted at the exact molecular abnormality associated with the neoplastic cell. Treatment with hematopoietic growth factors is used in some cases to stimulate leukemic cells to proliferate, making them more susceptible to cytotoxic drugs. This therapy also has been used to decrease the neutropenic, anemic, and thrombocytopenic period after chemotherapy or radiotherapy.

Review Questions

Level I

1. A gap in the normal maturation pyramid of cells with many blasts and some mature forms is known as: (Objectives 2, 5)
   A. leukemic hiatus
   B. chronic leukemia
   C. mixed cell lineage
   D. lineage restricted
2. Auer rods are inclusions found in: (Objective 5)
   A. myeloblasts
   B. lymphoblasts
   C. erythrocytes
   D. prolymphocytes
3. Chromosome changes in hematologic neoplasms are: (Objective 8)
   A. present in AL but not MPN or MDS
   B. nonrandom
   C. associated with a poor outcome
   D. not usually present
4. Genes that can cause tumors if activated are: (Objective 6)
   A. cancer genes
   B. proto-oncogenes
   C. preleukemia genes
   D. tumor suppressor genes
5. A common characteristic of acute lymphoblastic leukemia is: (Objective 2)
   A. *BCR/ABL1* gene mutation
   B. bone pain
   C. many blast cells with Auer rods
   D. leukocytopenia
6. Which of the following does the WHO classification use to subgroup the ALL into T- and B-cell neoplasms? (Objective 3)
   A. immunophenotype
   B. clinical presentation
   C. cytogenetics
   D. morphology
7. A leukemia that shows a profusion of granulocytes at all stages of development from blasts to segmented neutrophils is: (Objectives 2, 5)
   A. AML
   B. CML
   C. ALL
   D. CLL
8. Acute lymphoblastic leukemia occurs with greatest frequency in which age group? (Objective 7)
   A. 2–5 years
   B. 10–15 years
   C. 20–30 years
   D. >50 years
9. Chronic leukemias primarily affect: (Objectives 2, 7)
   A. all ages, progress rapidly, and have immature cells in peripheral circulation
   B. children, progress rapidly, and have mature cells in peripheral circulation
   C. young adults, progress slowly, and have immature cells in peripheral circulation
   D. adults, progress slowly, and have mature cells in circulation
10. The WHO system classifies a 19-year-old patient's bone marrow as a precursor B-cell leukemia. Which of the following best describes this leukemia? (Objectives 2, 3, 5)
    A. CLL
    B. ALL
    C. AML
    D. CML
11. Immunologic phenotyping of blast cells is important to: (Objective 8)
    A. help determine cell lineage
    B. identify the leukemia's etiology
    C. determine whether cytogenetic analysis is necessary
    D. replace the need to do multiple cytochemical stains
12. The minimum percentage of blast cells required for a diagnosis of acute myeloid leukemia using the WHO classification is: (Objective 3)
    A. 50%
    B. 40%
    C. 30%
    D. 20%
13. Proteins encoded by proto-oncogenes serve to: (Objective 10)
    A. provide signaling pathways for normal cell growth control
    B. inactivate tumor suppressor genes
    C. cause unregulated cell proliferation
    D. demethylate oncogenes

14. Which of these genes cause unregulated cell growth? (Objective 10)

    A. tumor suppressor genes

    B. antioncogenes

    C. proto-oncogenes

    D. oncogenes

Level II

1. The stain helpful in distinguishing myeloblasts from lymphoblasts is: (Objective 8)

    A. myeloperoxidase

    B. TRAP

    C. LAP

    D. esterase

2. If the cell of origin for a neoplastic tumor undergoes genetic mutations that gives the cell the ability to self-renew and blocks terminal differentiation, the resulting malignancy will be: (Objective 11)

    A. chronic myelogenous leukemia

    B. chronic lymphocytic leukemia

    C. acute leukemia

    D. myeloproliferative neoplasm

3. The PAS stain is helpful in diagnosing: (Objectives 3, 8)

    A. CLL

    B. CML

    C. MDS

    D. ALL

4. Which of the following factors has *not* been proposed as playing a role in causing leukemia? (Objective 6)

    A. benzene

    B. therapeutic radiation for Hodgkin disease

    C. living at high altitudes

    D. chromosome translocations

5. A 3-year-old child with Down syndrome presents with pallor, fatigue, lymphadenopathy, and hepatosplenomegaly. The initial CBC results were: (Objectives 3, 7)

WBC	18.7×10^9/L	**WBC Differential**
RBC	2.34×10^{12}/L	10% segs
Hb	5.8 g/dL (58 g/L)	27% lymphs
Hct	17.4% (0.174 L/L)	63% blasts
PLT	130×10^9/L	

    These findings are suggestive of:

    A. acute lymphoblastic leukemia

    B. chronic lymphocytic leukemia

    C. acute myeloid leukemia

    D. chronic myelogenous leukemia

6. A patient with a hypercellular, dysplastic bone marrow; anemia; and neutropenia in peripheral blood most likely has which of the following neoplasms? (Objective 7)

    A. MPN

    B. MDS

    C. AML

    D. ALL

7. A 52-year-old female was admitted to the hospital for minor elective surgery. Her pre-op CBC was:

WBC	49.4×10^9/L	**WBC Differential**
RBC	4.50×10^{12}/L	3% segs
Hb	12.7 g/dL (127 g/L)	97% lymphs
Hct	38% (0.38 L/L)	
PLT	213×10^9/L	

    What is the best explanation for the cause of this patient's leukocytosis and lymphocytosis? (Objectives 3, 7)

    A. ALL

    B. AML

    C. CLL

    D. CML

Use the following information to answer questions 8–10:

A 43-year-old male had been working with the Peace Corps in Mexico for the past 10 years. His primary responsibilities were taking radiographs and doing laboratory work at the various clinics. He had been complaining of weakness and fatigue for about a month and had had several severe nosebleeds. His CBC upon admission to the hospital was:

WBC	25.6×10^9/L	**Differential**
RBC	3.11×10^{12}/L	75% blasts
Hb	8.9 g/dL (89 g/L)	with Auer rods
Hct	26.7% (0.267 L/L)	20% lymphs
PLT	13×10^9/L	3% monos
		2% segs

8. Which leukemia is this patient most likely to have? (Objectives 3, 7)

    A. ALL

    B. AML

    C. CLL

    D. CML

9. What would be the most likely causative agent of the leukemia? (Objective 2)

    A. virus

    B. age

    C. hepatitis

    D. ionizing radiation

10. What treatment would result in the *best* prognosis for this patient if there were no complicating factors? (Objective 5)

    A. hematopoietic growth factors

    B. stem cell transplantation

    C. radiation therapy

    D. chemotherapy

11. You are doing several cytochemical stains on a bone marrow from a patient who was recently diagnosed with acute leukemia. You are looking at the MPO, and the blasts are negative. However, the reagent is nearing its expiration date, and you are not sure whether the stain worked properly. What cells found in the bone marrow normally express myeloperoxidase and could be used to assess the stain's integrity? (Objectives 8, 9)

    A. neutrophils

    B. red cell precursors

    C. megakaryocytes

    D. lymphocytes

Use this information to answer questions 12 and 13.

A pathologist is looking at the bone marrow aspirate of a 26-year-old male. The marrow is packed with undifferentiated blasts. After careful searching, she could not find any Auer rods. She thinks that the patient most probably has acute lymphoblastic leukemia but cannot rule out AML by using morphology.

12. The pathologist is thinking of ordering a TdT stain on the slides. Is that going to be useful? (Objectives 8, 9)

    A. Yes, TdT is always positive in acute lymphoid leukemia but never in myeloid leukemia.

    B. Yes, TdT is always positive in acute myeloid leukemia but never in lymphoid leukemia.

    C. No, TdT is positive in the majority of acute lymphoid leukemias, but approximately 20% of acute myeloid leukemias can be positive.

    D. No, TdT is positive in the majority of acute myeloid leukemias, but approximately 20% of acute lymphoid leukemias can be positive.

13. Which stain would you suggest the pathologist order? (Objectives 8, 9)

    A. PAS

    B. myeloperoxidase

    C. nonspecific esterase

    D. trichrome

14. Heritable changes in gene expression *not* from changes in DNA are known as: (Objective 10)

    A. chromosomal mutations

    B. pseudo-mutations

    C. epigenetic changes

    D. cytogenetic mutations

15. Mutations in tumor suppressor genes can result in: (Objective 1)

    A. inhibition of normal cell growth

    B. triggering growth-promoting signals in the absence of ligand binding

    C. activation of oncogenes

    D. tumor development

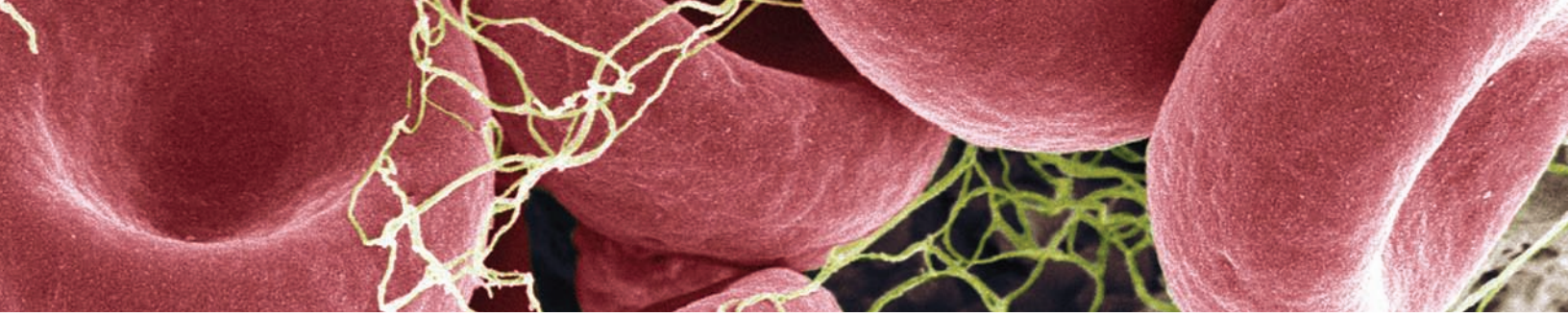
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Vardiman JW, Brunning RD, Arber DA et al. Introduction and overview of the classification of the myeloid neoplasms. In: *The International Agency for Research on Cancer. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue*. Washington, DC: IARC Press; 2008.
2. Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell*. 2012;21:283–96.
3. Passegue E, Jamieson CHM, Ailles LE et al. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci U S A*. 2003;100:11842–49.
4. Croce CE. Oncogenes and cancer. *N Engl J Med*. 2008;358:502–11.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–74.
6. Di Flore R, D'Anneo A, Tesoriere G et al. RB1 in cancer: different mechanisms of RB1 inactivation and alterations of pRb pathway in tumorigenesis. *J Cell Physiol*. 2013;228:1676–87.
7. Burkart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer*. 2008;8:671–82.
8. Bargonetti J, Manfredi JJ. Multiple roles of the tumor suppressor p53. *Curr Opin Oncol*. 2002;14:86–91.
9. Pietsch EC, Sykes SM, McMahon SB et al. The p53 family and programmed cell death. *Oncogene*. 2008;27:6507–21.
10. Malkin D. Li-Fraumeni syndrome. *Genes Cancer*. 2011;2:475–84.
11. Malkin D, Li FP, Strong LC et al. Germline p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science*. 1990;250:1233–38.
12. Esteller M. Epigenetics in cancer. *N Engl J Med*. 2008;358:1148–92.
13. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis*. 2010;31:27–36.
14. Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell*. 2012;150:12–27.
15. Guardavaccaro D, Pagano M. Stabilizers and destabilizers controlling cell cycle oscillators. *Mol Cell*. 2006;22:1–4.
16. Blain SW. Switching cyclin D-Cdk4 kinase activity on and off. *Cell Cycle*. 2008;7:892–898.
17. Nobori T, Miura K, Wu DJ et al. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*. 1994;368:753–56.
18. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*. 2007;26:1324–37.
19. Wojcik I, Szybka M, Golanska M et al. Abnormalities of the P53, MDM2, BCL2 and BAX genes in acute leukemias. *Neoplasma*. 2005;52: 318–24.
20. Xavier AC, Taub JW. Acute leukemia in children with Down syndrome. *Heamatologica*. 2010;95:1043-45.
21. Ross JA, Spector LG, Robison LL et al. Down syndrome and leukemia: state of the art series epidemiology of leukemia in children with Down syndrome. *Pediatr Blood Cancer*. 2005;44:8–12.
22. Gao Q, Horowitz M, Roulston D et al. Susceptibility gene for familial acute myeloid leukemia associated with loss of 5q and/or 7q is not localized on the commonly deleted portion of 5q. *Genes Chromosomes Cancer*. 2000;28: 164–72.
23. Darbary H, Stoler DL, Anderson GA. Family cancer syndromes: inherited deficiencies in systems for the maintenance of genomic integrity. *Surg Oncol Clin N Am*. 2009;18:1–17.
24. Dayaram T, Marriott SJ. Effect of transforming viruses on molecular mechanisms associated with cancer. *J Cell Physiol*. 2008;216:309–14.
25. Chan JK, Warner CG. Dynamic roles for NF-κB in HTLV-I and HIV-1 retroviral pathogenesis. *Immunol Rev*. 2012;246:286–310.
26. Jaffe ES, Harris NL, Stein H et al. *Pathology & Genetics. Tumours of Haematopoietic and Lymphoid Tissues. World Health Organization Classification of Tumours*. Washington, DC: IARC Press; 2001.
27. Kumar T, Mandla SG, Greer WL. Familial myelodysplastic syndrome with early age of onset. *Am J Hematol*. 2000;64:53–58.
28. Occhipinti E, Correa H, Yu I et al. Comparison of two classifications for pediatric myelodysplastic and myeloproliferative disorders. *Pediatr Blood Cancer*. 2005;44:240–44.
29. Kipps TJ, Wang H. Classification of malignant lymphoid disorders. In: Lichtman MA, Kipps TJ, Seligsohn U et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1403–408.
30. Brunning RD, Orazi A, Germing U et al. Myelodysplastic syndromes/neoplasms, overview. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC; 2008;87–107.
31. Paltiel O, Friedlander Y, Deutsch L et al. The interval between cancer diagnosis among mothers and off-spring in a population-based cohort. *Fam Cancer*. 2007;6:121–29.
32. Leukemia and Lymphoma Society. Leukemia facts and statistics. www.lls.org accessed November 10, 2013.
33. Pui C. Acute lymphoblastic leukemia. In: Kaushansky K, Lichtman MA, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010 (Chapter 93).
34. Bennett JM, Catovsky D, Daniel MT et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) Co-operative Group. *Br J Haematol*. 1976;33:451–58.
35. Vardiman JW, Thiele J, Arber DA et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–51.
36. Campo E, Swerdlow SH, Harris NL et al. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood*. 2011;117:5019–32.
37. National Committee for Clinical Laboratory Standards. *Proposed Standard: Histochemical Method for Leukocyte Alkaline Phosphatase*. Villanova, PA: Author; 1984.
38. Sigma Diagnostics. *Alkaline Phosphatase, Leukocyte: Histochemical Semiquantitative Demonstration in Leukocytes*. St. Louis: Sigma Diagnostics; 1990.
39. Yam LT, Li CY, Lam KW. Tartrate-resistant acid phosphatase isoenzyme in the reticulum cells of leukemic reticuloendotheliosis. *N Engl J Med*. 1971;284:357–60.
40. Yam LT, Janckila AJ, Li CY et al. Cytochemistry of tartrate-resistant acid phosphatase: fifteen years' experience. *Leukemia*. 1987;1:285–88.
41. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. *Blood*. 1979;54:1203–15.
42. Kung PC, Long JC, McCaffrey RP et al. Terminal deoxynucleotidyl transferase in the diagnosis of leukemia and malignant lymphoma. *Am J Med*. 1978;64:788–94.
43. Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. *Am J Clin Pathol*. 1971;55:283–90.
44. Li CY, Yam LT. Cytochemical characterization of leukemic cells with numerous cytoplasmic granules. *Mayo Clin Proc*. 1987;62:978–85.
45. Wick MR, Li CY, Pierre RV. Acute non-lymphocytic leukemia with basophilic differentiation. *Blood*. 1982;60:38–45.
46. Hughes T. ABL kinase inhibitor therapy for CML: baseline assessments and response monitoring. *Hematology Am Soc Hematol Educ Pro*. 2006;211–18.
47. Hughes T, Deininger M, Hochhaus A et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood*. 2006; 108:28–37.
48. Drucker BJ, Guilhot F, O'Brien SG et al. Five year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355:2408–17.
49. Ikeda A, Shankar DB, Watanabe M et al. Molecular targets and the treatment of myeloid leukemia. *Mol Genet Metab*. 2006;88:216–24.
50. Hehlmann R, Berger U, Pfirrmann M et al. Drug treatment is superior to allografting as first-line therapy in chronic myeloid leukemia. *Blood*. 2007;109:4686–92.
51. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128:683–92.
52. Garcia-Manero G, Issa J-P. Histone deacetylase inhibitors: a review of their clinical status as antineoplastic agents. *Cancer Invest*. 2005;23:635–42.
53. Garcia-Manero G. Modifying the epigenome as a therapeutic strategy in myelodysplasia. *Hematol Am Soc Hematol Educ Pro*. 2007;405–11.
54. Parsons SK. Oncology practice patterns in the use of hematopoietic growth factors. *Curr Opin Pediatr*. 2000;12:10–17.
55. Kaushansky K. Use of thrombopoietic growth factors in acute leukemia. *Leukemia*. 2000;14:505–8.



24 Myeloproliferative Neoplasms

TIM R. RANDOLPH, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Interpret the leukocyte alkaline phosphatase (LAP) activity found in patients with chronic myelogenous leukemia (CML).
2. Identify the major lineages involved with the various myeloproliferative neoplasms (MPNs): chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).
3. Recognize abnormal complete blood count (CBC) results that suggest an MPN.
4. Explain the diagnostic chromosome abnormality associated with CML and its significance in acute lymphoblastic leukemia (ALL).
5. Identify the peak incidence of CML according to age and sex distribution.
6. Describe and recognize the peripheral blood findings in CML patients and in those with blast crisis.
7. List and recognize laboratory findings typically associated with PMF.
8. Define criteria that indicate a transformation of an MPN into a blast crisis.
9. Compare the lab findings in primary PV to those in secondary polycythemia.
10. Describe and recognize the characteristic peripheral blood picture found in ET.
11. Describe and recognize the peripheral blood findings in patients with clonal hypereosinophila and mastocytosis.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Differentiate the subgroups of myeloproliferative neoplasms (MPNs) from other reactive and neoplastic diseases based on laboratory findings in the peripheral blood, bone marrow, and other diagnostic laboratory tests.
2. Contrast laboratory findings in MPNs and myelodysplastic syndromes (MDSs) as well as acute leukemia (AL).

(continued)

Chapter Outline

Objectives—Level II *(continued)*

3. Describe molecular gene mutations in MPNs and hypothesize how these changes in the pluripotential stem cell lead to MPNs and blast crisis (Philadelphia chromosome; *BCR/ABL1* rearrangement; *p53*, *p16* mutations; *JAK2* (V617F), *MPL* (W515), *FIP1L1/PDGFRA*, *ETV6-PDGFRB*, and *FGFR1* mutations and expression of growth factor receptors).
4. Differentiate CML from a benign leukemoid reaction using laboratory tests.
5. Assess the role of platelet-derived growth factor (PDGF) in the fibrosis associated with primary myelofibrosis (PMF).
6. Assess laboratory results including the evaluation of peripheral blood and bone marrow smears using the diagnostic criteria associated with PMF.
7. Compare clinical and laboratory findings, and interpret laboratory findings in relative polycythemia and absolute polycythemia.
8. Use laboratory results and clinical findings to differentiate essential thrombocythemia from nonmalignant conditions that result in thrombocytosis.
9. Use the criteria suggested for diagnosing variants of CML and chronic neutrophilic leukemia (CNL) to evaluate patients' laboratory results.
10. Differentiate unclassifiable myeloproliferative neoplasms from other subgroups of MPNs based on laboratory features.
11. Differentiate eosinophilic disorders using laboratory results and genetic mutations.
12. Describe systemic mastocytosis by giving the criteria for its diagnosis.
13. Using peripheral blood, bone marrow findings, and patient medical history, determine the classification of MPNs.

Key Terms

Blast crisis
Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
Chronic myelogenous leukemia (CML)
Chronic neutrophilic leukemia (CNL)
Clonal hypereosinophilia
Essential thrombocythemia (ET)
Hypereosinophilic syndrome (HES)
Janus kinase 2 (*JAK2*) gene
Leukemic hiatus
Panmyelosis
Plethora
Polycythemia vera (PV)
Primary myelofibrosis (PMF)
Spent phase
Systemic mastocytosis

Background Basics

The information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review these related concepts before starting this unit of study:

Level I

- Outline the cell cycle; describe stem cell differentiation and maturation for the various myeloid lineages. (Chapters 2, 3)
- Describe and recognize morphology for the various stages of myeloid maturation. (Chapter 7)
- Calculate red cell indices. (Chapters 10, 36)
- Use appropriate morphologic terms to describe size and chromia of red cells in anemic states. (Chapters 10, 11)
- Outline and explain the classification of hematopoietic neoplasms. (Chapter 23)
- Summarize the relationship of oncogenes to the neoplastic process (Chapter 23)

Level II

- Describe the influence of growth factors on hematopoietic cell proliferation. (Chapter 4)
- Explain the evaluation of red cell mass based on changes in fluid volume. (Chapter 11)
- Explain the hemoglobin–oxygen dissociation curve. (Chapter 6)
- Explain how proto-oncogenes are activated and their role in the etiology of hematopoietic neoplasms. (Chapter 23)
- Describe how cell marker panels can be used to differentiate hematopoietic neoplasms. (Chapters 23, 40)
- Discuss the value of cytogenetic studies in suspected hematopoietic neoplasms. (Chapters 23, 41)
- Explain how the utilization of molecular tests can assist in diagnosing hematopoietic neoplasms. (Chapters 23, 42)

CASE STUDY

We will refer to this case throughout the chapter.

Roger, a 52-year-old man with hyperuricemia, went to the clinic for a follow-up evaluation for splenomegaly. His palpable spleen, noted 18 months earlier, had been gradually enlarging. He originally denied fatigue, fever, and discomfort. He was examined, and a CBC was ordered. The results revealed leukocytosis, thrombocytosis, and anemia.

Consider how reflex laboratory testing can assist in diagnosing this patient.

OVERVIEW

This chapter presents the group of neoplastic but not truly malignant disorders called *myeloproliferative neoplasms (MPNs)*. MPNs must be distinguished from other neoplastic and benign hematologic disorders for the physician to select appropriate therapy for the patient. The chapter begins with the classification, pathophysiology, and general characteristics of MPNs, which provide the groundwork for a more detailed explanation and description of each subgroup. More specific pathophysiology, clinical findings, laboratory findings, and therapy are included for each subgroup. These are followed by an explanation of how to differentiate MPNs from diseases with similar laboratory findings. This is a lengthy chapter, so it is divided into

three sections to help the reader organize the study of this group of important disorders.

INTRODUCTION

Hematopoiesis is a highly regulated process whereby a normal steady-state production of hematopoietic cells in the bone marrow and destruction of senescent cells in the tissues maintain a constant peripheral blood cell concentration (Chapters 2, 3). Hematopoietic neoplasms result from acquired mutations in hematopoietic stem cells, allowing them to escape the regulatory controls for proliferation, natural cell death (apoptosis), and/or differentiation in the bone marrow (Chapter 23).

In the former French-American-British (FAB) classification system, neoplastic disorders of hematopoietic cells typically are grouped into three main categories: myeloproliferative disorders (MPDs), myelodysplastic states or syndromes (MDSs), and acute leukemias (ALs), including both myeloid and lymphoid subtypes. This classification system uses the blast count, lineage commitment, cell morphology, level of differentiation of the neoplastic cells, cytochemistry, and immunophenotyping to classify the diseases.

The World Health Organization (WHO) proposed a newer classification system for hematopoietic neoplasms that integrates cytogenetics, DNA analysis, and clinical features with cytochemistry and immunophenotyping. The WHO classification of myeloid disorders includes acute myeloid leukemia (AML), MPNs, MDSs, and the myelodysplastic/myeloproliferative neoplasms (MDS/MPN). The WHO's proposed MDS/MPN category includes neoplasms that are proliferative like MPNs but have dysplastic features like MDS. MDS/MPNs will be discussed in Chapter 25 with MDS. In addition, each group has subcategories based on clinical history, genetic, and various laboratory findings.

In the WHO classification, the percentage of blasts, degree of cell maturation, and dysplasia are critical assessments initially used to classify the hematopoietic neoplasms. A blast count ≥20% is necessary for an AL diagnosis, whereas the nonacute leukemia disorders have <20% blasts. MPNs and MDS are characterized by an autonomous, neoplastic clonal proliferation of hematopoietic precursors. Increased numbers of erythrocytes, leukocytes, and/or platelets in both the bone marrow and peripheral blood generally distinguish MPNs from MDS. MDS, on the other hand, is most commonly characterized by a hyperproliferative bone marrow, dysplastic maturation, and increased apoptosis that result in peripheral blood cytopenias. Both MPNs and MDS have the potential of evolving into acute leukemia.

PART I

OVERVIEW OF MYELOPROLIFERATIVE NEOPLASMS (MPNs)

CLASSIFICATION

The term *myeloproliferative syndrome*, coined by William Dameshek in 1951, describes a group of disorders that result from an unchecked, autonomous clonal proliferation of cellular elements in the bone marrow.[1] Under the WHO classification system, myeloproliferative neoplasms are generally characterized by panhypercellularity (**panmyelosis**) of the bone marrow accompanied by erythrocytosis, granulocytosis, and/or thrombocytosis in the peripheral blood. Although trilineage cell involvement (erythrocytic, granulocytic, thrombocytic) is characteristic of MPNs, one cell line is usually more prominently affected than the others. Hematologic classification is based on the most affected cell line (Table 24-1 ★). In the WHO classification,[2] the spectrum of myeloproliferative neoplasms includes:

- Chronic myelogenous leukemia (CML), Philadelphia (Ph) chromosome positive with *BCR-ABL1* fusion gene present, t(9;22)(q34;q12)
- Chronic neutrophilic leukemia (CNL), *BCR-ABL1*-negative
- Essential thrombocythemia (ET)
- Polycythemia vera (PV)
- Primary myelofibrosis (PMF, formerly known as *myelofibrosis with myeloid metaplasia* [*MMM*] and chronic idiopathic myelofibrosis [CIMF])
- Myeloproliferative neoplasm, unclassifiable (MPN-U)
- Myeloid and lymphoid neoplasms associated with eosinophilia and *PDGFRA*, *PDGFRB*, or *FGFR1* mutations
- Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
- Mastocytosis

The most important changes from the FAB to the WHO classification system of the MPNs are that (1) Ph chromosome must be present or if not identified by routine cytogenetic analysis, the *BCR/ABL1* fusion gene must be detected by molecular techniques for a diagnosis of CML, (2) minimum bone marrow blast count to distinguish MPNs from AL is 20% (MPNs <20% blasts), and (3) eosinophil disorders have been reclassified. Because identification of Ph is required for a diagnosis of CML, Ph-negative cases with myelodysplastic and

★ **TABLE 24-1** Classification of Myeloproliferative Neoplasms (MPNs) by Predominance of Cell Types

Involved Cell Line	Myeloproliferative Neoplasm
Myeloid	Chronic myelogenous leukemia (CML) and chronic neutrophilic leukemia (CNL), and sometimes primary myelofibrosis (PMF)
Megakaryocytic	Essential thrombocythemia (ET)
Erythroid	Polycythemia vera (PV)
Fibroblast[a]	Primary myelofibrosis (PMF)
Eosinophil	Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
Mast cell	Mastocytosis
Variable	Myeloproliferative neoplasm, unclassifiable (MPN, U)

[a]The fibroblast in PMF is not a part of the neoplastic process but is increased because of a reactive process.

★ **TABLE 24-2** Differential Features of Myeloproliferative Neoplasms

Parameter	CML	CNL	ET	PV	PMF	CEL-NOS
Peripheral blood:						
Hematocrit	N or ↑	N or ↑	N or ↓	↑↑↑	↓	N or ↓
Leukocyte	↑↑↑	↑	N or ↑	N or ↑	↑ or ↑↑	↑
Platelets	↑ or ↓	N or ↑	↑↑↑	↑	N,↑, ↓	N or ↓
Immature granulocytes	↑↑↑	Slight ↑(<10%)	Rare	Absent or ↑	↑↑	Absent or ↑
LAP	↓	↑	N or ↑	N or ↑	N,↑, ↓	N
Philadelphia chromosome	Present	Absent	Absent	Absent	Absent	Absent
Spleen size	N or ↑	↑	N or ↑	↑	↑↑↑	N
Bone marrow fibrosis	Absent or ↑	Absent	Absent or ↑	Absent or ↑	↑↑↑	Absent or ↑

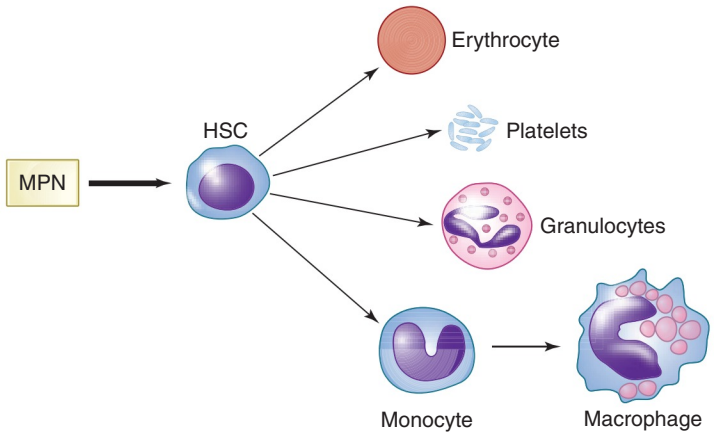
N = normal; ↓ = decreased; ↑, ↑↑, ↑↑↑ = slight, moderate, marked increase, respectively; LAP = leukocyte alkaline phosphatase; CML = chronic myelogenous leukemia; CNL = chronic neutrophilic leukemia; ET = essential thrombocythemia; PV = polycythemia vera; PMF = primary myelofibrosis; CEL-NOS = chronic eosinophilic leukemia, not otherwise specified

myeloproliferative features are included in the WHO MDS/MPN group and are called *atypical CML (aCML)*. The classification of these disorders is not always clear because of overlapping clinical and laboratory features between subgroups at different times during the disease course (Table 24-2 ★).

PATHOPHYSIOLOGY

The primary defect in MPNs appears to be in the pluripotential hematopoietic stem cell (HSC)[3] (Figure 24-1 ■). A clone of abnormal hematopoietic stem cells and their progeny preferentially expand until normal hematopoietic cell growth is inhibited and the majority of functioning bone marrow is derived from the abnormal clone. Excessive proliferation of hematopoietic cells occurs through various mechanisms that require multiple genetic mutations[3] (Chapter 23). Commitment, differentiation, and maturation of the abnormal clone are generally preserved, leading to increased numbers of mature cells in the peripheral blood, usually with one lineage (erythroid, myeloid, or megakaryocytic) predominating.

Finding uniform biochemical, cytogenetic, or molecular genetic abnormalities in hematopoietic cells from the bone marrow or peripheral blood of patients with MPNs suggests that these cells were derived from a single mutant stem cell (i.e., clonal origin) (Chapter 23). The abnormalities are not present in other somatic



■ **FIGURE 24-1** This schematic of hematopoietic cell development shows that a mutation in the pluripotential hematopoietic stem cell, HSC (CD34+), results in clonal proliferation of the progeny of that cell, all containing the mutation. Thus, all cell lines will be affected. The HSC is thought to be the original neoplastic cell in CML and other MPNs. Arrows indicate intermediate steps to mature cells that are not shown.

MPN = myeloproliferative neoplasm

cells, indicating that the mutations are acquired rather than inherited. The most consistent chromosome abnormality, the Ph chromosome, is found in all hematopoietic blood cells in patients with CML. Recently, the **Janus kinase 2 (*JAK2*) gene**, which codes for a tyrosine kinase involved in cell signaling, was found to be mutated in almost all cases of PV and some cases of ET and PMF. These mutations are discussed in more detail later in the chapter. The application of molecular biology in the study of hematopoietic neoplasms has been useful in identifying the specific genetic abnormality at the DNA, mRNA, and protein levels. These studies support the clonality hypothesis of MPNs.

GENERAL FEATURES

Myeloproliferative neoplasms usually occur in middle-aged and older adults (peak frequency in the fifth to seventh decades of life). They are rare in children. The onset of the disease is gradual, evolving over months or even years. The clinical, laboratory, and morphologic findings frequently overlap among the specific MPN disorders. Clinical findings can include hemorrhage, thrombosis, infection, pallor, and weakness. Anemia or polycythemia, leukoerythroblastosis, leukocytosis,thrombocytosis with bizarre platelet morphology, and bone marrow fibrosis are common laboratory findings that can occur in almost any of the MPN subtypes.

When present, anemia is caused by ineffective erythropoiesis, marrow fibrosis, and/or a shortened red cell survival because of splenic sequestration resulting in splenomegaly. While decreased bone marrow iron can be observed, it does not reflect true iron deficiency.[4] For this reason, serum ferritin and serum iron are more reliable estimates of iron deficiency in the presence of MPNs than is the evaluation of bone marrow iron.

Thrombocytosis can be present, and if it is classified as the primary disorder, it is believed to result from an autonomous, unregulated proliferation of megakaryocytes. The level of interleukin-6 (IL-6) and thrombopoietin (TPO), cytokines that promote megakaryopoiesis, is normal in MPNs, whereas the level is usually increased in secondary or reactive thrombocytosis.[5] Platelet membrane proteins (glycoprotein IIb/IIIa, von Willebrand factor [VWF], fibrinogen, fibronectin, and vitronectin) are significantly decreased in many patients with MPNs.[5] Genetic changes altering growth factor receptors, including the thrombopoietin receptor (myeloproliferative leukemia virus oncogene [*MPL*]), and mutations in the Janus kinase (*JAK*) signaling pathways result in increased megakaryopoiesis and enhanced activation and aggregation of platelets.[6]

The bone marrow is usually hypercellular at onset of the MPN but often becomes fibrotic during the course of the disease. Fibroblasts are a part of the bone marrow stroma, which provides a suitable microenvironment for developing hematopoietic cells (Chapters 3, 38). Fibroblasts produce reticular fibers that form a three-dimensional supporting network for vascular sinuses and hematopoietic elements. *Fibrosis* refers to an increase in fibroblasts and reticular fibers. Fibrosis in MPNs is thought to be a reactive process that is secondary to the increased production of cytokines from the abnormal hematopoietic cells (primarily megakaryocytes). As evidence of their benign proliferation, fibroblasts exhibit normal karyotypes. Although often considered the hallmark of PMF, fibrosis can be seen in the other MPNs as well.

Reactive fibrosis can result from the intramedullary release of cytokines from platelets, megakaryocytes, and malignant cells that are mitogenic for fibroblasts. Human platelet-derived growth factor (PDGF) stimulates growth and cell division of fibroblasts as well as other cells. The platelet concentration of PDGF in patients with MPNs is significantly decreased, likely because of excessive release. The serum PDGF level is significantly higher in patients with PMF and ET than in other MPNs or in normal controls. Not surprisingly, PMF and ET are the two MPNs with the most significant degree of fibrosis.[5] Increased fibroblast proliferation and function leads to an increase in collagen, laminin, and fibronectin in the medullary cavity.

When marrow fibrosis supervenes over the course of the disease, the major sites of hematopoiesis become the extramedullary tissues, particularly the liver and spleen. Hepatosplenomegaly is a common clinical finding. Extramedullary hematopoiesis can also occur in benign diseases such as chronic hemolytic anemias, but the hematopoiesis in these disorders is confined to the erythrocyte lineage. In contrast, all cell lineages are present in the extramedullary masses that accompany the MPN. Marrow fibrosis likely causes distortion of marrow sinusoids, which permit HSCs to escape into the sinusoids and gain entry to the peripheral blood.[7] The HSCs then lodge in extramedullary sites, such as the spleen, to proliferate and differentiate.

MPNs carry a significant risk of terminating in acute leukemia. This transition may result from chromosome instability in the original mutant stem cell[8] or as the result of leukemogenic chemotherapy and radiotherapy for the original myeloproliferative neoplasm (Chapter 23).

☑ CHECKPOINT 24-1

In essential thrombocythemia, all hematopoietic lines have increased cell proliferation. Which lineage has the greatest increase?

PART II

SUBGROUPS OF MPNs

CHRONIC MYELOGENOUS LEUKEMIA (CML)

Chronic myelogenous leukemia (CML), also known as *chronic granulocytic leukemia (CGL)*, *chronic myeloid leukemia*, and *chronic myelocytic leukemia*, is the best-defined MPN. It is characterized by a neoplastic growth of primarily myeloid cells in the bone marrow with an extreme elevation of these cells in the peripheral blood. Erythrocytic and megakaryocytic lineages can also expand and extramedullary granulocytic proliferation in the spleen and liver reflects progression of the disease.

The natural course of the disease occurs in three phases: chronic, accelerated, and **blast crisis**. The initial chronic phase responds well to therapy. Use of traditional chemotherapy can usually restore and maintain normal health for months or years. CML can eventually

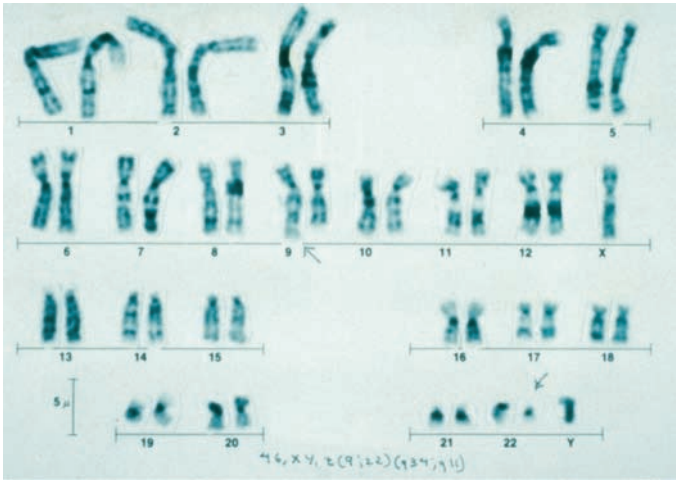
transform into an accelerated phase and then into blast phase or acute leukemia. The acute leukemic phase can be either acute myeloid leukemia (AML) or lymphoblastic leukemia (LL, also called acute LL [ALL]). After progression to the blast crisis phase, the prognosis is poor with a survival of <6 months using traditional chemotherapy. However, 70–90% of patients who are treated in the chronic phase with tyrosine kinase inhibitors (imatinib mesylate) experience a 5-year progression-free survival.

Etiology and Pathophysiology

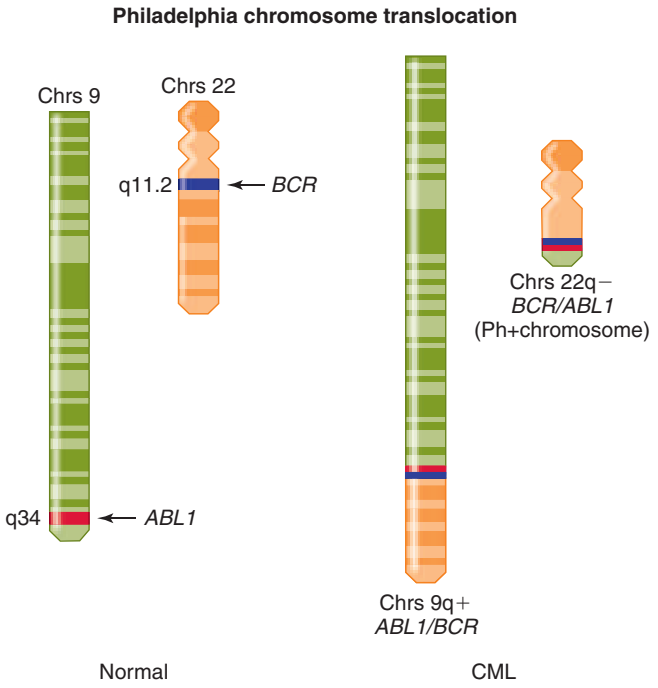
The Ph chromosome (Ph) is an acquired chromosomal translocation that results in a fusion gene called *BCR/ABL1*. This fusion gene is present in all neoplastic hematopoietic cells in CML except T lymphocytes and sometimes B lymphocytes and is necessary for the diagnosis of CML (Figure 24-2 ■) (Chapters 41, 42). The Ph chromosome was the first chromosome abnormality found consistently associated with a malignant disease. It is not found in other somatic cells or fibroblasts.

In some cases, the Ph chromosome can be detected months before the diagnosis of CML. Once the chromosome is identified, it will rise and fall as a reflection of tumor burden and can be used as a measure of disease progression, remission, and relapse. Because all hematopoietic cells are involved in the neoplastic process (evidenced by the presence of Ph in those cells), the original neoplastic cell (cell of origin) is most likely a pluripotential HSC (Figure 24-1).

Molecular biology has dramatically increased our understanding of the role of the Ph in this disease. The Ph results from a balanced reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11.2) (Figure 24-3 ■). On chromosome 9, the breakpoint is spread across a 90 kb region within the first exon of *ABL1*, translocating the 3′ end of exon 1 and exons 2–11 (in which the tyrosine kinase domains reside) to chromosome 22. The breakpoint in chromosome 22 occurs within the *BCR* gene, a large 70-kb gene with 25 exons. The breakpoint usually occurs in an area known as the *major breakpoint cluster region* (*M-BCR*). When the breakpoint occurs in the M-BCR, two different fusion genes are formed. Both fusion genes join the translocated portion of the *ABL1* gene (exons 2–11) with either exon 13 or 14 of *BCR*. The resulting Ph has a 5′ BCR

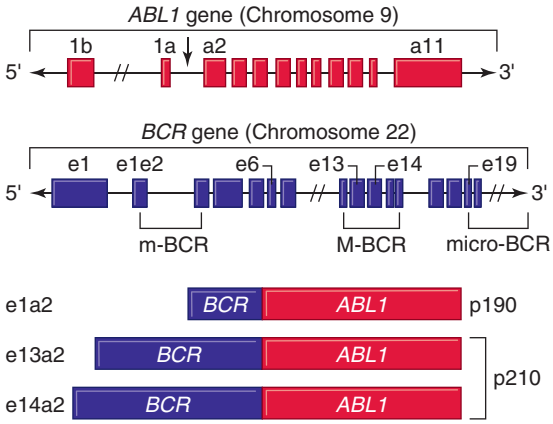


■ **FIGURE 24-2** Karyotype from a patient with CML showing the Ph chromosome translocation, t(9:22)(q34;q11.2). The Ph chromosome is chromosome 22.



■ **FIGURE 24-3** The Philadelphia translocation in CML. Arrows indicate the chromosome breakpoints at 9q34 (*ABL1* gene) and 22q11.2 (*BCR* gene) in the genes directly involved in the translocation. The translocation results in a lengthened 9q+ chromosome and a shortened 22q− chromosome (Ph chromosome).

head (exons 1-13 or 1-14) from chromosome 22 and a 3′ *ABL1* tail (exons 2–11) from chromosome 9 (Figure 24-4 ■). This hybrid gene is then transcribed into an 8.5-kb fusion mRNA, in contrast to the normal *ABL1*, which is transcribed to a 6- or 7-kb mRNA.[9]



■ **FIGURE 24-4** Exon maps of the normal *BCR* and *ABL1* genes, and the *BCR/ABL1* fusion gene formed by the Ph chromosome translocation found in CML and de novo acute leukemias. Breakpoints in the *ABL1* gene occur upstream of exon 2 (a2, downward arrow). Breakpoints in the major breakpoint cluster region (M-BCR) of the *BCR* gene occur in exon 13 (e13) and exon 14 (e14) in CML; breakpoints can also occur in the minor breakpoint cluster region (m-BCR, 3′ of exon1 [e1]) in acute leukemias. Following *BCR/ABL1* fusion, the resulting protein of 210 (p210; e13a2 or e14a2) or 190 (p190; e1a2) kD is formed.

★ **TABLE 24-3** Characteristics of the Proto-Oncogenes and Oncogenes Involved in the *BCR/ABL1* Gene Rearrangements in CML

Gene	Size	No. Exons	Breakpoint	Transcript(s)	Protein	Location	Activity
ABL1	>230 kb	11	5′ of exon II (100–200 kb)	6 kb; 7 kb	p145	Nucleus	Tyrosine kinase (normal)
BCR	>100 kb	25	BCR (5.8 kb)	4.5 kb; 6.7 kb	p160	Cytoplasm	Serine/Threonine kinase
BCR/ABL1 fusion	Varies	Varies	Fusion hybrid	8.5 kb	p210	Plasma membrane	Tyrosine kinase (increased)

Translation of the 8.5-kb fusion mRNA creates a new, abnormal fusion protein, with a molecular mass of 210 kD (p210, normal ABL1 is a 145-kD protein, Figure 24-4). This fusion protein produces constitutive tyrosine kinase activity, increasing autophosphorylation within the cell that activates intracellular signal transduction pathways, and results in abnormal gene expression (Chapters 2, 23). Tyrosine kinase enzymes regulate metabolic pathways by transferring phosphate groups from phosphate donors (ATP and GTP) to target proteins at tyrosine residues, activating the protein. Some serve as receptors for growth factors (RTK [receptor tyrosine kinase]). The oncogenic role of p210 is found in association with increased granulocyte colony–stimulating factor (G-CSF) and PDGF and suppression of apoptosis in hematopoietic cells.[9] Over a period of several years, the t(9;22) cell line replaces the normal marrow cells, and the clinical presentation of CML is observed. The reciprocal fusion gene, *ABL1/BCR1*, is transcribed in approximately two-thirds of CML patients, although its significance is not clear. Characteristics of the *ABL1* and *BCR* proto-oncogenes, as well as the *BCR/ABL1* fusion oncogene, are described in Table 24-3 ★.

Detection of the *BCR* gene rearrangement has several clinical uses in the diagnosis and prognosis of CML (Table 24-4 ★).[9,10] About 5–10% of patients with the CML phenotype lack Ph. In these cases, the translocation is not detected at the karyotypic level by cytogenetic studies but is detected at the molecular level by reverse transcriptase polymerase chain reaction (RT-PCR) or fluorescent in situ hybridization (FISH), verifying that the molecular *BCR/ABL1* fusion has occurred (Chapters 41, 42). The malignant cells in these cases also express the 8.5-kb chimeric mRNA and the p210 protein (Table 24-5 ★).

Cases of phenotypic CML that are both Ph chromosome negative and *BCR/ABL1*-negative are usually CNL, aCML, or possibly cases of chronic myelomonocytic leukemia (CMML) if absolute monocytosis and leukocytosis are present. The last two disorders are now considered MDS/MPN but all have high proliferative rates.[11]

★ **TABLE 24-4** Clinical Uses of Molecular Analysis for the *BCR/ABL1* Gene Rearrangement[10]

- Differential diagnosis of CML
- Diagnosis of CML when Ph1 is absent
- Differentiation of CML in blast crisis from de novo ALL when Ph1 is present
- Confirmation of a diagnosis of CML when the patient presents in the blast crisis phase of CML
- Monitoring of CML patients on tyrosine kinase inhibitor (TKI) therapy to determine response, resistance, or relapse
- Detection of minimal residual disease in post-bone marrow transplant patients

Disease Progression and Additional Chromosome/Molecular Mutations

Historically, or in untreated cases, progression of chronic CML is marked by an accelerated phase followed by an acute phase (blast crisis). In about 80% of patients, this progression is preceded or accompanied by the development of additional chromosomal abnormalities (Table 24-6 ★).[9] Thus, repeated chromosome analysis in patients with CML can be helpful in predicting disease progression.

At the molecular level, mutations in the *p53* gene, a tumor suppressor gene, are found in at least 25% of patients in blast crisis, especially in those with myeloid blast crisis,[9] while mutations/alterations of *p53* in the chronic state of CML are rare. Other tumor suppressor genes such as *p16* and *RB* and oncogenes such as *BCL-2* and *AML1* can also acquire mutations that contribute to the development of blast crisis (Chapter 23).[9]

Philadelphia (Ph) Chromosome in Acute Leukemias

About 2–5% of childhood ALL, 25% of adult ALL, and some cases of AML have the Ph chromosome at diagnosis (Chapters 25, 26). Ph-positive AML cases can actually be CML in blast crisis that were not diagnosed in the chronic (CML) stage. In about 50% of the Ph-positive ALLs, the BCR/ABL1 protein, p210, is present and these cases

★ **TABLE 24-5** Genetic Rearrangements and Related Proteins Found in Philadelphia Chromosome Positive CML, ALL, and Philadelphia Chromosome Negative MPN, or ALL

Clinical Condition	Involvement of Ph/BCR	Size of TK
Normal	Ph−, BCR−	145 kD
CML	Ph+, BCR+	210 kD
	Ph−, BCR+	210 kD
Other MPNs	Ph−, BCR−	145 kD
ALL	Ph+, BCR+	210 kD (CML blast crisis)
	Ph+, BCR− (minor BCR+)	190 kD (? de novo ALL)
	Ph−, BCR−	145 kD (? de novo ALL)
	Ph+, BCR− (micro BCR+)	230 kD (variant CML)

TK = tyrosine kinase; CML = chronic; ALL = acute; Ph+ = Philadelphia; Ph− = Philadelphia; BCR+ = rearrangement, BCR− = norearrangement within the M-BCR region

★ **TABLE 24-6** Additional Chromosomal Changes in Blast Crisis of CML

Rate of Occurrence	Chromosomal Change
Frequent	Duplication of Ph chromosome
	Trisomy 8
	Isochromosome 17
	Loss of Y chromosome
Rare	Translocation (15;17)
	Translocation (3;21)(q26;q22)
	Translocation (3;3)/inversion (3)
Very rare	Deletion of chromosome 5(−5) or the long arm (5q−)
	Deletion of chromosome 7(−7) or the long arm (7q−)

probably represent the blast crisis phase of CML. In the remaining 50% of Ph-positive ALL, the breakpoints on chromosome 22 fall 5′ to the M-BCR within the first intron of the *BCR* gene (minor breakpoint cluster region, m-BCR). These leukemias express a distinct translation product from the mRNA hybrid termed the p190 kD protein[9] (Figure 24-4). These Ph-positive ALLs may actually be de novo acute leukemia cases (Table 24-5). Like p210, the p190 protein also shows an increased tyrosine kinase activity, but its role in the development of the ALL phenotype is unclear.

☑ CHECKPOINT 24-2

A patient has the CML phenotype, but the genetic karyotype does not show the Ph chromosome. If this is truly a CML, what should molecular analysis show?

Clinical Findings

CML is the most common MPN, accounting for 15–20% of all leukemia cases. It can occur at any age, but the incidence increases dramatically among those 55 years of age and older. It is most prevalent in the seventh, eighth, and ninth decades of life and is almost equally distributed between sexes. CML can occur in young adults more so than other MPNs. Although rare, CML can occur in childhood. The incidence of CML is highest in countries that are more economically advanced. The question arises, however, as to whether the higher incidences reflect the ability to detect and diagnose the disease because of increased availability of advanced medicine and diagnostic tools in these countries versus a higher mutagen exposure.

The disease has an insidious onset with the most common symptoms being increased weakness, loss of stamina, unexplained fever, night sweats, weight loss, and feelings of fullness in the abdomen (hepatosplenomegaly). Gastrointestinal tract bleeding or retinal hemorrhages are occasionally the first signs of the disease. Physical examination reveals pallor, tenderness over the lower sternum, splenomegaly, and occasionally hepatomegaly. Lymphadenopathy is not typical but when present, it suggests an onset of the acute phase of the disease. Petechiae and ecchymoses reflect the presence of quantitative and/or qualitative platelet abnormalities. Some individuals are asymptomatic, and CML is found incidentally during examination for other medical problems or during a routine physical examination.

Any organ eventually can be infiltrated with myeloid elements, but extramedullary masses in areas other than the spleen and liver are uncommon findings in the chronic phase. On fresh incision, extramedullary masses appear green, presumably because of the presence of the myeloid enzyme myeloperoxidase. These greenish tumors have been called *chloromas*. The green color fades to a dirty yellow when the tissue is exposed to air.

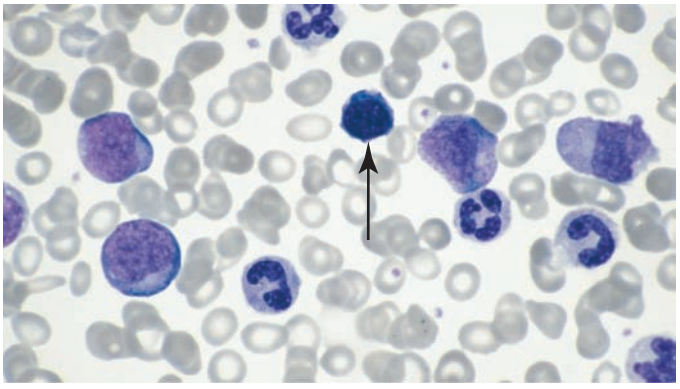
Without intervention, symptoms worsen over the next 3–5 years, and increased debilitation heralds the onset of the blast phase. With the onset of blast crisis, response to therapy is poor and survival is <6 months using traditional chemotherapy. Treatment with tyrosine kinase inhibitors has dramatically improved patient outcomes.

Laboratory Findings

Peripheral Blood

The most striking abnormality in the peripheral blood is the extreme leukocytosis. The white count is usually >100 × 10^9/L with a median of about 170 × 10^9/L Patients diagnosed early may have a leukocyte count of 25–75 × 10^9/L Thrombocytosis, which can exceed 1000 × 10^9/L, and variation in platelet shape are found in more than half of the patients. If thrombocytosis is a new observation, blast crisis is probably imminent; however, during blast crisis, thrombocytopenia is a common finding. Platelet function also is frequently abnormal. Megakaryocyte fragments and micromegakaryocytes can be found (Figure 24-5 ■).

At the time of diagnosis, a mild to moderate normocytic, normochromic anemia is typical with a hemoglobin concentration in the range of 9–13 g/dL. The severity of anemia is proportional to the increase in leukocytes. Erythrocyte morphology is generally normal, but nucleated erythrocytes can be found. Reticulocytes are normal or slightly increased.



■ **FIGURE 24-5** Arrow points to a micromegakaryocyte in the peripheral blood of a patient with CML (peripheral blood, Wright-Giemsa stain, 1000× magnification).

Blood smears exhibit a shift to the left with all stages of granulocyte maturation present (Figure 24-6 ■). The predominant cells are the segmented neutrophils and myelocytes. Promyelocytes and blasts do not usually exceed 20% of the leukocytes in the peripheral blood. Eosinophils and basophils are often increased in both relative and absolute terms. Increasing blast or basophil numbers herald blast crisis. Monocytes are moderately increased. Signs of myeloid dysplasia including pseudo–Pelger-Huët anomaly (hyposegmentation of the neutrophil nucleus [Chapter 21]) and decreased leukocyte alkaline phosphatase (LAP) are frequent (Chapters 23, 37). Low or absent LAP is characteristic but not specific for CML. Monocytosis, myeloid dysplasia, and micromegakaryocytes are overlapping features found in both CML and chronic myelomonocytic leukemia (CMML), but the presence of the Ph will differentiate the two disorders.

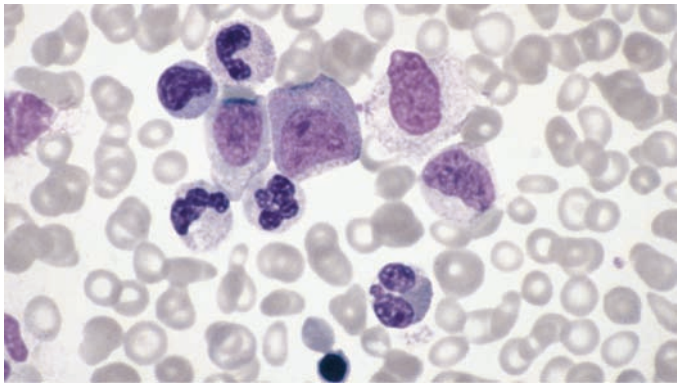
Bone Marrow

The bone marrow is 90–100% cellular with a striking increase in the myeloid-to-erythroid ratio (10:1 to 50:1) reflective of the myelopoiesis. The active red marrow can extend into the long bones. Cortical thinning and erosion of the trabeculae can be present. The hematopoietic marrow cells are primarily immature granulocytes with <20% blasts by WHO classification, an important characteristic that distinguishes CML from all forms of acute leukemia (AL).[2] The marrow differential count of leukocyte precursors often is within the reference interval. The typical **leukemic hiatus** (lack of developing cells between immature cells [blasts] and mature cells [segmented neutrophils]) that is characteristic of AL is not present in CML. Auer rods can be found in the myeloblasts during blast crisis, but this is an unusual finding. Erythropoiesis is normoblastic because the increased tyrosine kinase activity does not directly affect erythropoiesis, but normoblasts can be decreased. Megakaryocytes are usually increased with frequent immature and atypical forms. In contrast to the large megakaryocytes found in other subgroups of MPNs, CML typically reveals small megakaryocytes.

Gaucher-like cells (histiocytes exhibiting a wrinkled tissue paper appearance of the cytoplasm) have been observed in the bone marrow (Chapter 21). However, these Gaucher-like cells do not occur because of the lack of the β-glucocerebrosidase enzyme as in Gaucher's disease but because of the overload of cerebrosides caused by increased cell turnover. The histiocytes in CML have normal to increased amounts of β-glucocerebrosidase, but the cells cannot process the excess cerebrosides fast enough to prevent them from accumulating.

The marrow can become fibrotic late in the course of the disease. If the patient does not see a physician until the fibrosis is prominent, an inappropriate diagnosis of PMF could be considered. At this point, chromosomal (Ph) or molecular (*BCR/ABL1*) analysis can establish the correct diagnosis.

Other nonspecific findings related to the increased proliferation of cells can be present. Total serum cobalamin and the unsaturated cobalamin binding capacity are increased. Serum haptocorrins are often elevated (Chapter 15). These findings are probably related to the increased number of granulocytes that are thought to synthesize these proteins. Uric acid and lactic dehydrogenase (LD) are elevated, secondarily to increased cell turnover. Muramidase is normal or only slightly increased.



■ **FIGURE 24-6** CML with leukocytosis and a shift to the left (peripheral blood, Wright-Giemsa stain, 1000× magnification).

☑ CHECKPOINT 24-3

Describe the peripheral blood differential of a CML patient.

CASE STUDY (continued from page 447)

Physical examination revealed a slightly enlarged liver and palpable spleen. Roger had hyperuricemia. Blood counts showed:

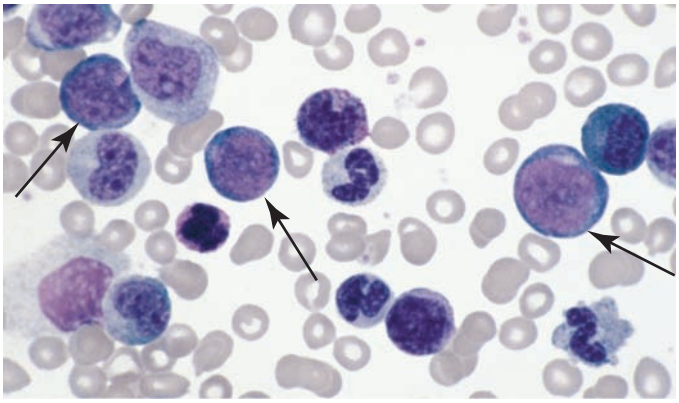
Hb	11.6 g/dL
Hct	0.35 L/L (35%)
RBC	3.6×10^{12}/L
WBC	26.2×10^{9}/L
Platelets	853×10^{9}/L

The blood cell differential showed marked anisocytosis, poikilocytosis with many teardrops, and numerous nucleated red blood cells (NRBCs). Immature myeloid cells were found along with basophilia and large platelets.

1. What are Roger's MCV and MCHC?
2. How would you classify his anemia morphologically?
3. Based on Roger's history and current laboratory data, what other tests should be performed?

Terminal Phase

If untreated, the typical CML course progresses to the accelerated stage approximately 3–5 years after diagnosis (Figure 24-7 ■). Transition to the accelerated phase is heralded by one or more of the changes listed in Table 24-7 ★, can occur at any time after the initial diagnosis, and often precedes the final stage of blast transformation (blast crisis). Clinical features reflect an increase in debilitation including pyrexia (fever), night sweats, weight loss, increased weakness, malaise, bone pain, and lymphadenopathy. About 30% of those patients in the accelerated phase die before developing blast crisis.



■ **FIGURE 24-7** Peripheral blood film from a patient with CML in accelerated phase. There is an increase in blasts. Arrows point to blasts (Wright-Giemsa stain, 1000× magnification).

Although blast crisis typically develops after a short accelerated phase, about one-third of cases abruptly develop a blast transformation. After onset of blast crisis, survival is about 1–2 months using classical chemotherapy and is dramatically improved for most patients receiving TKI therapy. The clinical features in blast crisis are similar to those of acute leukemia (Chapters 26, 27).

The hematologic criteria for identifying blast crisis is made by finding moderate to marked diffuse bone marrow fibrosis and ≥20% blasts[2] in the peripheral blood or bone marrow of a patient previously diagnosed as having CML (Table 24-8 ★ and Figure 24-8 ■). Any type of blast involvement is possible including myeloid, lymphoid, erythroid, or megakaryocytic cells. Because blast morphology alone is often not sufficient to identify the type of blast involved, cytochemical, enzymatic, ultrastructural, and immunophenotyping studies are necessary (Chapters 23, 37, 40).

About 65–75% of blast crises are myeloblastic, and 25–35% of blast crises are lymphoblastic. The lymphoblasts in blast crisis are immunologically typed as common acute lymphocytic leukemia antigen positive (CALLA+/CD10+) (Chapters 8, 27, 40) and demonstrate an elevated terminal deoxynucleotidyl transferase (TdT) suggesting that these cells belong to the B lymphocyte lineage (Chapter 8). Erythroblastic and megakaryoblastic crises are uncommon.

Therapy

The purpose of CML therapy is to reduce the leukocyte mass, restore bone marrow function, reduce splenomegaly, and abolish symptoms. Leukapheresis is sometimes used initially to reduce the leukocyte mass when excessive numbers of cells result in a significant increase in blood viscosity. Supportive measures during therapeutic regimens include transfusion to treat severe anemia and antibiotics to treat infections.

Before the discovery of molecularly targeted therapy for malignant diseases, the use of interferon-α, plus cytarabine was considered standard therapy for patients with CML who were not candidates for an allogeneic HSC transplant. Interferon-α, a glycoprotein, has a myelosuppressive effect directly inhibiting myeloid progenitor cells. It induces a remission in 55–75% of CML patients and, in some cases, eliminates the Ph clone. A cytogenetic response is more favorable in patients who are treated early in the chronic phase of the disease.[9]

Imatinib

Currently, the tyrosine kinase inhibitor (TKI) imatinib mesylate (Gleevec®), is considered the first treatment option except in pregnant patients. It is a molecular-targeted therapy. Imatinib competitively binds to the ATP binding site of the tyrosine kinase of *ABL1* (*BCR/ABL1* and *TEL/ABL1*), stem cell factor receptor (c-Kit), and platelet-derived growth factor receptor (PDGFR), thus inhibiting kinase activity and preventing cell signal transduction. Imatinib is well tolerated with mild to moderate side effects, primarily involving the gastrointestinal tract and skin. However, it is teratogenic in laboratory animals. Results of a 12-year study using imatinib indicate that 83% of patients have a complete hematologic response by 12 months and 96% at 60 months.[12] Imatinib also has been shown to be effective in

★ **TABLE 24-7** Accelerated Phase of CML

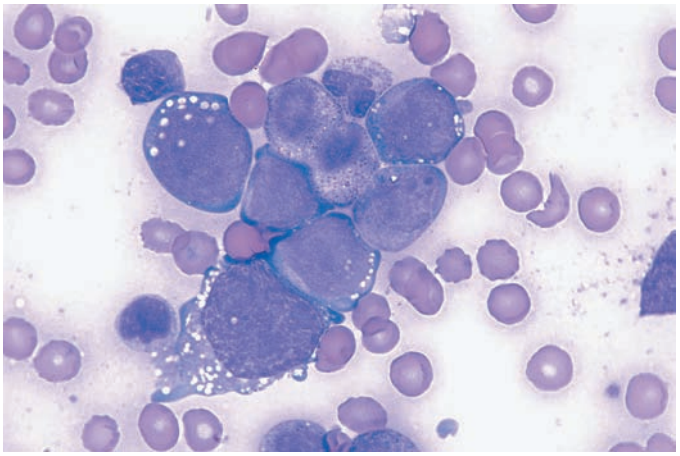
Characterized by the presence of one or more of the following:

- >10% myeloblasts in peripheral blood or bone marrow
- ≥20% basophils in peripheral blood
- Persistent thrombocytopenia $<100 \times 10^9$/L (unrelated to therapy)
- Persistent thrombocytosis $>1000 \times 10^9$/L (unresponsive to therapy)
- Increasing WBC count and/or spleen size (unresponsive to therapy)
- Additional clonal genetic aberrations not present in the chronic phase

★ **TABLE 24-8** Blast Crisis Phase of CML

Characterized by the presence of one or more of the following:

- ≥20% blasts in the bone marrow or nucleated cells in the peripheral blood
- Extramedullary blast proliferation
- Presence of large clusters of blasts in the bone marrow biopsy specimen



■ **FIGURE 24-8** Peripheral blood from a patient with CML in blast crisis. Note the cluster of blasts with vacuoles (Wright-Giemsa stain, 1000× magnification).

improving molecular and cytogenetic markers and delaying progression of disease in patients in blast crisis.[12,13]

Patients initially responsive to imatinib therapy can develop resistance (secondary resistance or loss of response), whereas other patients might not respond to initial therapy (primary resistance; Table 24-9 ★).[14] The majority of patients in the advanced stages of CML show a measurable response to imatinib although fewer of them reach remission compared with those treated in the chronic phase.

Imatinib Resistance

In the majority of cases, loss of response (secondary resistance, Table 24-9) is related to development of additional mutations in the kinase domain of *BCR/ABL1*.[9] A particular ABL1 protein mutant, T315I, in which threonine is mutated to isoleucine at the gatekeeper position of the kinase domain does not respond to any of the TKIs currently used in CML therapy and represents about 15% of imatinib resistant patients.[15] Mutation screening is suggested for those who do not have an adequate initial response or who experience a loss of response to imatinib because the position of the mutation within the kinase domain can be clinically relevant. Some mutations have inferior survival (P-loop of BCR/ABL1 protein kinase mutations) compared with mutations at other sites.[16]

Several therapeutic approaches for patients who experience imatinib resistance exist. Dose escalation is often the first course of action. Remission can be recovered by imatinib dose escalation when the loss of remission is caused by the development of a second Ph in the HSC or *BCR/ABL1* gene duplication. Occasionally, relapse because of certain mutations in the ATP binding site can be overcome through imatinib dose escalation.[16]

When dose escalation fails, switching to an alternative tyrosine kinase inhibitor is the treatment of choice. Dasatinib (BMS354825) is a tyrosine kinase inhibitor that, like imatinib, attaches to the ATP binding site of the ABL1 portion of the BCR/ABL1 protein. It is effective against all imatinib resistant mutants currently identified except the T315I mutation.

Nilotinib (AMN107) is a structural derivative of imatinib that binds to and stabilizes the ABL protein ATP binding site with 30-times greater potency than imatinib. Like dasatinib, nilotinib is effective against all imatinib resistant mutants currently identified except the T315I mutation.

★ **TABLE 24-9** Resistance to Imatinib in Patients with CML

Resistance	Definition
Primary resistance	• Complete hematologic response is not achieved by 3–6 months • Cytogenetic response is not achieved by 6 months (>95% Ph+ metaphases persist) • Partial cytogenetic response is not achieved by 12 months (>35% Ph+ metaphases persist)
Secondary resistance	• Loss of a complete hematologic response • Loss of a partial or complete cytogenetic response • Increasing levels of *BCR/ABL1* as assessed by real-time PCR

In September 2012, the U.S. Food and Drug Administration approved bosutinib for the treatment of chronic, accelerated, or blast phase Philadelphia chromosome positive (Ph+) chronic myelogenous leukemia (CML) in adult patients with resistance or intolerance to prior therapy. Like dasatinib and nilotinib, it is effective against most imatinib-resistant mutants except the T315I mutation. Preliminary results suggest that second generation TKIs could be superior first-line therapies over imatinib for new CML patients.[16]

Baseline assessment for CML patients prior to starting imatinib therapy should include bone marrow morphologic analysis, cytogenetics, and RT-PCR to determine baseline level of BCR/ABL1 transcripts. Measuring BCR/ABL1 mRNA levels in plasma has been found to correspond with patient tumor burden and is suggested as a monitoring standard for patients.[17] The RT-PCR assay of blood should be performed at least every 3 months after therapy begins, or a single assay at 6 months can be performed if the patient is responding.[18] Bone marrow cytogenetics should be performed every 6 months until a major cytogenetic response (MCyR, <35% Ph+), is achieved. A complete cytogenetic response (0% Ph+) and *BCR/ABL1*-negative results (complete molecular response) in treated patients predict a favorable outcome. Incomplete response (primary resistance) indicates the presence of minimal residual disease, and increasinging BCR/ABL1 results indicate the potential for disease relapse (secondary resistance).

HSC Transplantation

Allogenic HSC transplantation can be an option for patients who meet the bone marrow transplant criteria. Limitations are based on patient age (<65 years) and availability of a compatible donor.[9] High-dose chemoradiotherapy or imatinib therapy is followed by HSC transplantation from syngeneic or allogenenic donors. Stem cell transplantation is most successful when administered during the first chronic phase of CML. Of patients receiving HLA-matched sibling stem cells, 86% have survival rates that exceed 3 years.[9]

Differential Diagnosis

Many infectious, inflammatory, or malignant disorders and severe hemorrhage or hemolysis can cause a leukemoid reaction (Chapter 21) that resembles CML (Table 24-10 ★). At times, the clinical findings of a leukemoid reaction permit an accurate diagnosis, but in some cases, differential diagnosis requires further investigation. In a leukemoid reaction, leukocytosis is generally accompanied by a predominance of segmented neutrophils and bands on the blood smear (myelocytes, metamyelocytes, promyelocytes, and blasts are few in number compared with CML). Toxic granulation, cytoplasmic vacuoles, and Döhle bodies in granulocytes often accompany benign toxic leukocytosis in leukemoid reactions and are not common in CML. Monocytes, eosinophils, and basophils are generally not elevated in a leukemoid reaction in contrast to CML.

Other diagnostic tests (elevated LAP score and absence of Ph) are helpful in differentiating a leukemoid reaction from CML. Splenomegaly is uncommon in a leukemoid reaction. Although a bone marrow examination is rarely necessary to make a differential diagnosis, the marrow in a leukemoid reaction can be hypercellular, but in contrast to CML, the maturation of granulocytic cells is orderly.

★ **TABLE 24-10** Comparison of Peripheral Blood Features of CML and Leukemoid Reactions

Laboratory Parameter	CML	Leukemoid Reaction
Leukocytes	Moderate to marked leukocytosis, blasts and promyelocytes in peripheral blood (deep left shift), toxic changes usually absent, eosinophilia and basophilia, neutrophils with single-lobed nuclei and hypogranular forms can be present	Mild to moderate leukocytosis, toxic granulation, Döhle bodies and vacuoles present, blasts absent, promyelocytes rare, no absolute basophilia or eosinophilia
Platelets	Often increased with abnormal morphological forms present, occasional micromegakaryocytes	Usually normal
Erythrocytes	Anemia usually present, variable anisocytosis, poikilocytosis, NRBC present	Anemia rarely, NRBC not typical
LAP	Low	Increased
Chromosome karyotype	Ph or *BCR/ABL1* translocation present	Normal

CML occasionally resembles PMF. Distinguishing features of PMF include markedly abnormal erythrocyte morphology with nucleated erythrocytes and immature leukocytes (leukoerythroblastic reaction), an increased LAP score, bone marrow fibrosis, and the absence of the Ph.

CNL is another MPN that may resemble CML but does not have the *BCR/ABL1* mutation. It must be distinguished from CML because in the absence of the *BCR/ABL1* mutation, imatinib is not an effective treatment. Because of the similarity of CNL to CML, it is discussed next.

☑ CHECKPOINT 24-4

What clinical, peripheral blood, and genetic features differentiate CML from an infectious process?

CHRONIC NEUTROPHILIC LEUKEMIA (CNL)

Chronic neutrophilic leukemia (CNL) is an MPN characterized by a sustained increase in neutrophils in the peripheral blood with a slight shift to the left and no Ph or *BCR/ABL1* mutation. Monocytosis and basophilia are absent, which helps to distinguish CNL from myelodysplasia and CML, respectively. The bone marrow is hypercellular because of increased neutrophilic granulocyte proliferation, but dysplasia is absent. All causes of a reactive neutrophilia and other MPNs must be ruled out for a diagnosis of CNL, so it is a diagnosis of exclusion. The diagnostic criteria for CNL[19] are defined in Table 24-11 ★.

Etiology and Pathophysiology

The cause of CNL has not been identified. About 20% of cases are associated with an underlying neoplasm, particularly multiple myeloma. However, clonality of the neutrophils when neutrophilia is associated with multiple myeloma has not been demonstrated. Thus, neutrophil proliferation may be related to abnormal cytokine production in this setting. The CNL cell of origin cells is probably a bone marrow stem cell with limited myeloid lineage potential. Normal apoptotic signaling is likely disrupted.

Cytogenetics are normal in most patients, but up to 25% have mutations including 20q−, 21+, 11q−, and +8, +9. Ph and *BCR/ABL1* fusion gene are absent. If plasma cell proliferation is present, clonality of the neutrophils should be established by cytogenetic or molecular studies for a diagnosis of CNL.

Clinical Findings

CNL is a rare MPN, with approximately 150 reported cases. The median age at diagnosis is 65 years. The male-to-female ratio is about 1:1. Most patients are asymptomatic at the time of diagnosis, but fatigue, weight loss, easy bruising, bone pain, and night sweats can occur. Hepatosplenomegaly is usually present.

★ **TABLE 24-11** Diagnostic Criteria for Chronic Neutrophilic Leukemia

Peripheral blood leukocytosis ≥25 × 10^9/L
- Bands and segmented neutrophils >80% white blood count (WBC)
- Immature granulocytes <10% WBC
- Myeloblasts <1% WBC

Hypercellular BM with increased percent of neutrophilic granulocytes,
- <5% myeloblasts
- Normal neutrophil maturation pattern
- Normal or left shift of megakaryocytes

Hepatosplenomegaly

No cause identified for physiologic neutrophilia
- Absence of infection/inflammation
- No underlying tumor

Absence of Ph chromosome or *BCR/ABL1* mutation

Absence of mutations of *PDGFRA*, *PDGFRB*, or *FGFR1*

No evidence of other MPNs

No evidence of an MDS or MDS/MPN disorder; monocytes <1 × 10^9/L; no dysplastic changes in granulocytes or other myeloid cells

Adapted from: Bain BJ, Brunning RD, Vardiman JW, Thiele J. Chronic neutrophilic leukemia. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon, France: IARC Press; 2008:38.

Laboratory Findings

The most notable feature is neutrophilia (>25 × 10^9/L) (Figure 24-9 ■). Mature segmented forms and bands predominate, and more immature cells account for <10% of the leukocytes. Neutrophils can appear toxic but not dysplastic. Both the RBC count and platelet morphology are normal. Platelets are usually present in normal concentration, but thrombocytopenia can develop as the disease progresses and the spleen enlarges. Bone marrow is hypercellular with an M:E ratio that can reach 20:1 or higher. Granulocytic hyperplasia is present, but dysplasia, Auer rods in blasts, and increased blasts are absent. Excessive erythroid and megakaroycytic proliferation can be present. The LAP score is usually increased.

Therapy

Hydroxyurea is a first-line therapy for CNL. Response lasts for about 12 months. Second-line therapy is interferon-α. Allogeneic stem cell transplantation is a potentially curative treatment for those patients who are eligible.

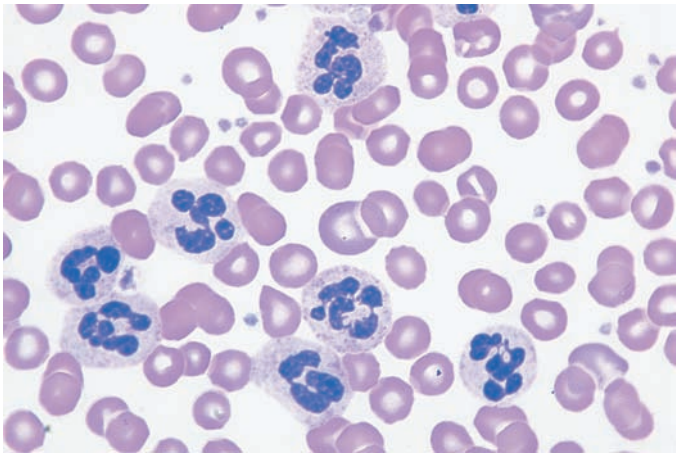
Median survival is 2.5 years. CNL also has an accelerated phase that is marked by progressive neutrophilia unresponsive to treatment, anemia, thrombocytopenia, and splenomegaly.[9] Blasts and other immature cells can be present in the peripheral blood.

Differential Diagnosis

CNL must be differentiated from CML and physiologic causes of neutrophilia such as infection or inflammation. Differential diagnosis from other myeloid neoplasms requires an absence of circulating blasts, absolute monocytosis, eosinophilia, and basophilia.

☑ CHECKPOINT 24-5

What is the most important feature that separates all other forms of MPNs from CML?



■ **FIGURE 24-9** Peripheral blood from a patient with CNL. Note the increased numbers of mature neutrophils (Wright-Giemsa stain, 1000× magnification).

ESSENTIAL THROMBOCYTHEMIA (ET)

Essential thrombocythemia (ET) is a myeloproliferative neoplasm affecting primarily the megakaryocytic lineage. Sustained proliferation of megakaryocytes in the marrow and extreme thrombocytosis in the peripheral blood with thrombocytopathy (a qualitative disorder of platelets) occurs. Previously, considerable controversy existed concerning the inclusion of ET as a specific entity in the myeloproliferative disorders because thrombocytosis is often a component of CML, PMF, and PV. However, ET is now firmly established as a hematologic neoplasm with distinct clinical manifestations and complications.[20]

Synonyms of ET include *primary thrombocythemia*, *hemorrhagic thrombocythemia*, *primary thrombocytosis*, and *idiopathic thrombocytosis*.

Etiology and Pathophysiology

ET is a neoplastic disorder of the HSCs usually resulting in clonal hematopoiesis affecting all three lineages, but in some cases, involving only the megakaryocytes.[20] The clonal population of cells appears hypersensitive to some cytokines, including IL-3 and IL-6, but the clones are not hypersensitive to GM-CSF. Sensitivity to the inhibitory effects of TGF-β is decreased, minimizing inhibition of thrombopoiesis. Thus, a combination of increased sensitivity to some cytokines that promote platelet production coupled with a decreased sensitivity to negative regulators could account for the increased megakaryocyte proliferation characteristic of ET.

TPO and/or its receptor MPL are not commonly associated with the underlying pathology of ET. Expression of MPL and its mRNA are generally decreased in ET, serum levels of thrombopoietin are normal or slightly elevated in most patients, yet proliferation of progenitor cells ensues.[20]

JAK2(V617F)

The normal JAK2 protein is a cytoplasmic protein kinase closely associated with cytokine receptors and thus is distributed almost exclusively near the cell membrane. When a receptor binds cytokines, the JAK2 protein is transphosphorylated and activated. In turn, JAK2 phosphorylates **s**ignal **t**ransducers and **a**ctivators of **t**ranscription (STAT) 5 proteins. The JAK–receptor complex also activates other signaling pathways. Several inhibitory control mechanisms constrain the normal JAK2/STAT5 activation pathway. The JAK2 protein has two homologous domains: JH1, which has functional (kinase domain) activity, and JH2, which lacks kinase activity (pseudo-kinase domain). The JK2 domain normally interacts with the JH1 domain to inhibit kinase activity and to modulate or regulate receptor signaling.[20]

In 2005, a gain-of-function mutation in the *JAK2* tyrosine kinase gene, *JAK2*(V617F), was identified in patients with PV and later in ET and PMF.[20] *JAK2*(V617F) is found on chromosome band 9p24 and encodes a mutated tyrosine kinase activator of cell signal transduction. The single nucleotide somatic mutation in exon 14 (substitution of thymine for guanine) results in the substitution of the amino acid valine for phenylalanine at position 617. The amino acid substitution occurs in the pseudo-kinase JK2 domain of the JAK2 protein. This reduces the JH2 inhibitory function and results in constitutive activation. Furthermore, the mutation allows for downstream

phosphorylatation of STAT5 molecules independent of cytokine interaction with the receptor (i.e., autonomous signaling; Chapter 3). This *JAK2*(V617F) gain-of-function mutation gives the cells a proliferation advantage.

Presence of the *JAK2*(V617F) mutation appears to increase the risk of thrombosis proportional to the degree of leukocytosis with no correlation to platelet count.[21] The connection between leukocytosis and thrombosis may be the result of tissue factor expression by neutrophils, platelet P-selectin expression, and aggregate formation between platelets and leukocytes that lead to interaction with the endothelium in areas of inflammation and injury.[22,23]

A single *JAK2*(V617F) mutation is more commonly encountered in ET, whereas two *JAK2*(V617F) mutations are associated with PV.[24–27] This dosage effect is associated with disease transformation from ET to PV and with disease progression in patients with PV. Therefore, *JAK2*(V617F) burden is implicated as having a dominant role in the pathophysiology of Ph negative MPNs.[28,29] The mutation also results in increased responsiveness to erythropoietin (EPO) and IL-3. *JAK2*(V617F) is found in ~50% of patients with PMF and most patients (>95%) with polycythemia vera.[20,30,31]

Mutations in cytokine receptors for EPO, TPO, and G-CSF may activate JAK2 in those cases that are *JAK2*(V617F) negative.

MPL Mutations

Two mutations in the thrombopoietin receptor *MPL* (*MPL*W515L and *MPL*W515K) occur in approximately 4% of patients with *JAK2*(V617F)-negative ET and 10% with PMF. This mutation results in cytokine-independent growth and constitutive downstream signaling pathways.[20]

Clinical Findings

Although a relatively rare disorder, the incidence of ET peaks primarily from 50–60 years of age and secondarily from 20–30 years of age. The older group of patients has no gender predilection, but the younger age group predominantly involves women. The overall incidence is ~1.5–2.4/100,000 people annually.[20]

The presenting symptoms of patients with ET are variable. Extreme thrombocytosis is frequently detected. Many of these patients are asymptomatic, and their diagnosis is made incidentally.[20] Symptomatic patients most commonly present with thrombosis (primarily involving the microvasculature) or minor bleeding. Neurologic complications are common (e.g., headache, paresthesias of the extremities) and are associated with platelet-mediated ischemia and thrombosis. Circulatory insufficiency involving the microvasculature of the toes and fingers is frequent and associated with pain and occasionally gangrene. Hemorrhagic episodes can occur, primarily involving the gastrointestinal tract, skin, urinary tract, and oral mucosal membranes. These problems appear to be more frequent in patients older than 59 years of age with thrombosis occurring more frequently than bleeding at the lower platelet concentrations.[20,32]

About half of the patients have a palpable spleen, but splenomegaly is usually slight. Occasionally, splenic atrophy resulting from repeated splenic thrombosis and silent infarctions is seen. When this occurs, it is associated with typical morphologic alterations on the peripheral blood smear as discussed in the next section.

Laboratory Findings

Peripheral Blood

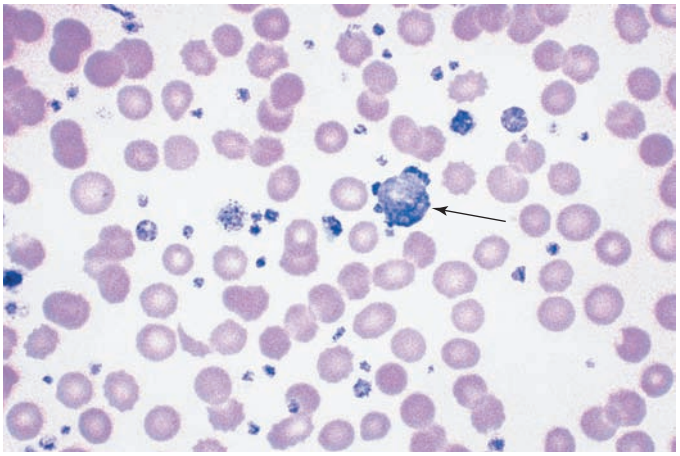
The most striking finding in the peripheral blood is extreme and consistent thrombocytosis (Figure 24-10 ■). Platelet counts are $>450 \times 10^9$/L and often range from $1000–5000 \times 10^9$/L. The peripheral blood smear can show giant bizarre platelets, and platelets can appear in aggregates. Megakaryocytes and megakaryocyte fragments also can be found. However, in many cases, platelet morphology appears normal. Abnormalities in platelet aggregation and adhesion suggest defects in platelet function (Chapter 36).

Anemia, if present, is generally proportional to the severity of bleeding and is usually normocytic; however, long-standing or recurrent hemorrhagic episodes can lead to iron deficiency and a microcytic, hypochromic anemia. In about one-third of the patients, slight erythrocytosis is present and can cause confusion with polycythemia vera. Aggregated platelets can lead to an erroneous increase in the erythrocyte count on automated cell counters. Therefore, hemoglobin determinations are better to assess the patient's anemic status. The reticulocyte count can be increased if bleeding is present in which case mild polychromatophilia may be noted. Peripheral blood abnormalities secondary to autosplenectomy can occur if the spleen has been infarcted (Chapter 3). These abnormalities include Howell-Jolly bodies, nucleated erythrocytes, and poikilocytosis.

A leukocytosis from $22–40 \times 10^9$/L is almost always present. Occasional metamyelocytes and myelocytes can be found with ET. Mild eosinophilia and basophilia also are observed. The LAP score can be normal or increased; it is rarely low. Nucleated erythrocytes are found in 25% of patients.

Bone Marrow

The bone marrow exhibits marked hyperplasia with a striking increase in megakaryocytes often with clustering of megakaryocytes along the sinusoidal borders. The background of stained slides shows many platelets. The megakaryocytes are large with abundant mature cytoplasm and frequently increased nuclear lobulation. Mitotic forms are



■ **FIGURE 24-10** Essential thrombocythemia. Platelets are markedly increased, and a giant form (arrow) is present (peripheral blood, Wright-Giemsa stain, 1000× magnification).

increased. Erythroid and myeloid hyperplasia also are evident. Stains for iron reveal normal or decreased stores in the context of normal serum ferritin levels. In ~25% of cases, reticulin is increased, but significant fibrosis is generally not seen.[20]

Tests of Hemostasis

Laboratory tests alone are unreliable in predicting bleeding or thrombotic complications in ET. The prothrombin time (PT) and activated partial thromboplastin time (APTT) are usually normal, but evidence of low-grade disseminated intravascular coagulation (DIC) can be present. Platelet aggregation studies are frequently abnormal with the most common findings including defective platelet aggregation with epinephrine, ADP, and collagen. A loss of platelet α-adrenergic receptors associated with reduced epinephrine-induced aggregation is characteristic of an MPN and is useful in differentiating ET from secondary thrombocytosis. Spontaneous in vitro platelet aggregation, or hyperaggregability, is a common finding. In vivo platelet aggregation is likely if increased plasma β-thromboglobulin and platelet factor 4 (released from platelet α-granules) levels are found. Other platelet abnormalities that have been described in association with ET are included in Table 24-12 ★.[33] A form of acquired von Willebrand disease (VWD) has been described in association with excessively high platelet counts and ET. The increase in number of circulating platelets is associated with adsorption of larger VWF multimers and their removal from the circulation. The laboratory features are characteristic of Type 2 VWD with a decrease or absence of large VWF multimers and reduced levels of ristocetin cofactor activity (Chapters 34, 36).

Molecular Genetics

A low incidence of clonal chromosomal cytogenetic abnormalities (about 5%) is found in ET. The *JAK2*(V617F) gene mutation confers a polycythemia-like phenotype often producing higher hemoglobin values, leukocytosis, and lower erythropoietin levels, increasing the risk of transformation to PV.[22,23] A small percentage of patients carry an *MPL* gene with mutations in exon 10, the most common of which is the *MPL*(W515L).

Other Laboratory Findings

Other laboratory tests can be abnormal. Serum cobalamin and the unsaturated cobalamin binding capacity are increased. An increase in cell turnover can cause serum uric acid, lactate dehydrogenase (LD), and acid phosphatase to be elevated. Serum potassium can be elevated as a result of in vitro release of potassium from platelets (pseudohyperkalemia). The spurious nature of this hyperkalemia can be verified by performing a simultaneous potassium assay on plasma, which should be normal. Arterial blood gases can reveal a pseudohypoxia if the sample is not tested promptly because of the in vitro consumption of oxygen by the increased numbers of platelets.

★ **TABLE 24-12** Platelet Abnormalities Found in Essential Thrombocythemia

- Decreased or mutated MPL receptors for thrombopoietin (TPO)
- Shortened platelet survival
- Increased plasma β-thromboglobulin (β-TG)
- Increased urinary thromboxane B_2 ($T \times B_2$)
- Acquired Von Willebrand disease
- Defective epinephrine, collagen, ADP-induced platelet aggregation
- Decreased ATP secretion
- Acquired storage pool deficiency because of abnormal in vivo platelet activation

Prognosis and Therapy

About 76–89% of the patients with ET survive 10 years. The prognosis appears to be better in younger patients. The most common causes of death are thrombosis and bleeding. Occasionally, the disease transforms to AML or PMF.

Controversy exists as to which patients with ET require therapy. It is generally agreed that patients with a history of thrombosis or cardiovascular risk factors require therapy to reduce the platelet count. Plateletpheresis can quickly reduce the platelet count below 1000×10^9/L for control of vascular accidents. Anticoagulants and drugs to inhibit platelet function are used to control thrombosis, and aspirin therapy is recommended for all ET patients unless contraindicated.[20]

Chemotherapy

Cytoreductive therapy frequently includes use of hydroxyurea and anagrelide. Hydroxyurea is an effective form of cytoreductive therapy in patients to lower both the leukocyte and platelet counts and lower the risk of thrombosis. Although anagrelide (inhibitor of megakaryocyte differentiation) can be used to reduce only the platelet count, its side effects are not well tolerated.[20] The leukemogenic potential of these therapeutic agents is of concern. The benefit of specific therapy in asymptomatic patients has not been established. Therapeutic trials with β-interferon show improvement in both hematologic parameters and clinical symptoms on nearly all patients.[24] Withdrawal of interferon, however, leads to recurrence of thrombocytosis.

Molecular Targets

Use of JAK inhibitors is being investigated primarily in PMF but is beginning to be studied in patients with advanced ET and PV. These include ICNB018424 (ruxolitinib) and CEP-701 (lestaurtinib).[34,35] Patient response to lestaurtinib is not as promising as that for ruxolitinib.[36]

Differential Diagnosis

Although the other MPNs have certain diagnostic markers, ET is largely a diagnosis of exclusion. Essential thrombocytosis must be differentiated from a secondary, reactive thrombocytosis (Table 24-13 ★) associated with many acute and chronic infections, inflammatory diseases, carcinomas, and Hodgkin's disease. The platelet count in ET often exceeds 1000×10^9/L and is persistent over a period of months or years. Secondary or reactive thrombocytosis rarely reaches 1000×10^9/L and is transitory. In addition, platelet function in secondary thrombocytosis is normal as are leukocytes and erythrocytes, and splenomegaly is absent.

★ **TABLE 24-13** Conditions Associated with Thrombocytosis

Essential thrombocythemia (ET)
Polycythemia vera (PV)
Chronic myelogenous leukemia (CML)
Primary myelofibrosis (PMF)
Secondary thrombocytosis
- Chronic inflammatory disorders
- Acute hemorrhage
- Hemolytic anemia
- Hodgkin's disease
- Metastatic carcinoma
- Lymphoma
- Postsplenectomy
- Postoperative
- Iron deficiency

Differentiating ET from PV can be difficult. However, marked erythrocytosis with clinical findings suggestive of hypervolemia is more typical of PV. The Polycythemia Vera Study Group (PVSG) proposed a set of diagnostic criteria for ET, which were adopted as diagnostic criteria in the WHO classification (Table 24-14 ★). The first criterion, a platelet count of >600 × 10^9/L, later reduced by the WHO group to 450 × 10^9/L, excludes many cases of secondary thrombocytosis. The second criterion, increased megakaryocytes in the bone marrow, is necessary to confirm the diagnosis of ET and rule out other causes of clonal thrombocythemia. The third, a hemoglobin of <18.5 g/dL in males and <16.5 g/dL in females excludes cases of PV as does the fourth criterion, presence of iron in the bone marrow or failure of response to iron therapy. The fifth criterion, absence of the Ph, rules out CML, and the sixth, absence of collagen fibrosis, rules out PMF. The seventh criterion, absence of dysplasia and related genetic mutations, rules out MDS. The eighth criterion excludes conditions associated with reactive thrombocytosis.[37]

★ **TABLE 24-14** Essential Thrombocythemia Diagnostic Criteria (all criteria must be met)[37,38,39]

Diagnosis requires meeting all four criteria:
1. Sustained platelet count ≥450 × 10^9/L
2. Megakaryocyte proliferation with large and mature megakaryocytes without an increase or left-shift in neutrophil granulopoiesis or erythropoiesis
3. WHO criteria for CML, polycythemia vera, primary myelofibrosis, MDS or other myeloid neoplasm are not met
4. Presence of an acquired pathogenic mutation such as *JAK2*(V617F) or other clonal marker
5. No evidence of reactive thrombocytosis and normal iron stores

☑ CHECKPOINT 24-6

Is a patient who has a platelet count of 846 × 10^9/L, splenomegaly, and abnormal platelet function tests of hyperaggregation likely to have reactive or essential thrombocytosis? Explain.

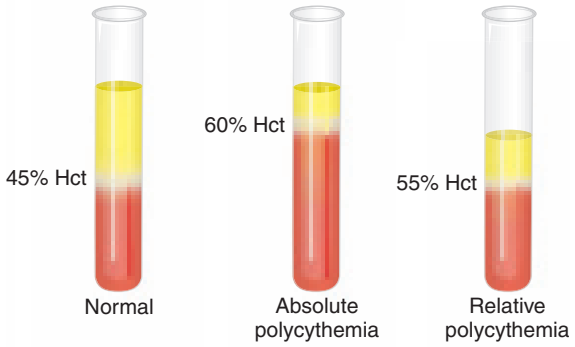
POLYCYTHEMIA VERA (PV)

The term *polycythemia* literally means an increase in the cellular elements of the blood. However, it is most commonly used to describe an increase in erythrocytes exclusive of leukocytes and platelets. **Polycythemia vera (PV)** is a myeloproliferative neoplasm characterized by an unregulated proliferation of primarily the erythroid elements in the bone marrow and an increase in erythrocyte concentration in the peripheral blood. In addition to autonomous proliferation of erythroid cells, leukocytes and platelets can also proliferate uncontrolled, resulting in a pancytosis (an increase in all hematopoietic cells in the blood). PV has several synonyms, including *polycythemia rubra vera*, *primary polycythemia*, *erythremia*, and *Osler's disease*.

Classification

Polycythemia is a general term used to describe erythrocytosis resulting in an increase in both hemoglobin concentration and red cell mass (RCM) or hematocrit (Chapter 10). When evaluating a patient for polycythemia, it is important to determine whether these parameters are elevated because of an absolute increase in total erythrocyte mass (absolute erythrocytosis) or from a decrease in plasma volume (relative erythrocytosis) (Figure 24-11 ■). Although an absolute erythrocytosis suggests a PV diagnosis, polycythemia that is secondary to tissue hypoxia, cardiac or pulmonary disease, and abnormal hemoglobins should also be considered.

In an attempt to clarify the pathogenesis of the disorder, polycythemia is classified into three different groups: PV, secondary



■ **FIGURE 24-11** The hematocrit can be increased because of an absolute increase in erythrocyte mass, a condition known as *absolute polycythemia* (center) or a decrease in plasma volume, a condition known as *relative polycythemia* (right).

polycythemia, and relative polycythemia (Table 24-15 ★). Both PV and secondary polycythemia result from an absolute increase in the total body RCM. Secondary polycythemia can be distinguished from PV by a distinct, although not always apparent, physiologic stimulus of erythrocytosis—hence, the name *secondary polycythemia*—and is associated with elevated plasma EPO levels. PV results from a primary, unregulated, or dysregulated increase in erythrocyte production. Relative polycythemia is characterized by a normal or even decreased RCM and occurs as a result of a decreased plasma volume. It is generally a mild polycythemia resulting from dehydration, hemoconcentration, or a condition known as *Gaisböck's syndrome*.

Etiology and Pathophysiology

The panhyperplasia often associated with PV suggests a clonal stem cell defect, and cytogenetic studies have confirmed its clonal nature.[40] Evidence of clonality persists in cells even during complete remission.

Although all lineages in the peripheral blood can be increased in PV, an increase in erythropoiesis is the outstanding feature. Possible mechanisms for this increase are suggested in Table 24-16 ★.[41] In vitro studies using cell culture systems show that PV bone marrow cells can form erythroid colonies without the addition of erythropoietin, suggesting that increased proliferation results from an unregulated neoplastic proliferation of stem cells.[40] Other patients' bone marrow cells show increased sensitivity to EPO, insulin-like growth factor I, and IL-3, forming in vitro colonies at significantly reduced cytokine concentrations and likely giving PV progenitor cells a growth advantage.[42,43] The erythroid maturation is morphologically normal and the erythrocytes function normally and have a normal lifespan.

JAK2

Several mutations, most affecting the Janus kinase–STAT signal transduction pathway, have been identified in patients with PV. The *JAK2*(V617F) mutation seen in almost all patients (>95%) with PV is found on chromosome band 9p24 and encodes a mutated tyrosine kinase activator of cell signal transduction.[40] In addition, about 5% of PV cases carry other mutations in exon 12 of *JAK2* that disrupt normal *JAK2* function.[44]

Since the *JAK2*(V617F) is not found in all PV patients, it is hypothesized that *JAK2*(V617F) is not the sole transforming mutation, and many suspect it is not even the initiating event.[45,46] This theory is supported by evidence that in some patients with *JAK2* mutated myeloproliferative neoplasms that evolved into AML, the resulting myeloblasts do not bear the *JAK2* mutation.[47] Therefore, one or more mutations are likely to precede the *JAK2*(V617F) mutation and are necessary to predispose patients to developing the *JAK2*(V617F) mutation and the resulting MPN.

Two mutations that may precede the *JAK2*(V617F) mutation include *TET2* (ten-eleven translocation) and a germline single nucleotide polymorphism (SNP) in the *JAK2* locus (rs10974944).[48] The TET2 protein functions as a catalyst in the hydroxylation of 5-methylcytosine (5-mC), converting 5-mC to 5-hydroxylmethylcytosine (5-hmC).[49] It is thought that 5-hmC is an intermediate base in the demethylation of DNA and therefore serves as an epigenetic regulator that participates in driving myeloid proliferation (Chapter 23).[50] Mutations that delete or lead to loss of function in *TET2* are thought to occur in the HSC and contribute to myeloid rather than lymphoid neoplasms.[40] Clonal analysis of immature progenitor cells in patients with *JAK2* and *TET2* mutations suggest that *TET2* precedes *JAK2* with the exception of one report of familial MPN for which the opposite is suspected.[51]

A germline polymorphism in the *JAK2* gene (rs10974944) is found between exons 12 and 13. This polymorphism increases the likelihood of developing the *JAK2*(V617F) mutation and the resulting MPN by three- to fourfold.[52]

Other Mutations

In the absence of the *JAK2*(V617F) mutation, other mutations have been identified that disrupt the normal function of the JAK/STAT pathway resulting in myeloid proliferation. These include *MPL*,[40] the lymphocyte-specific adapter protein LNK,[53] and an anti-apoptotic protein member of the Bcl-2 family called Bcl-x_L.[43]

MPL mutations promote thrombopoietin independent activation of the MPL protein and the resulting thrombocytosis associated with ET and PV. Furthermore, the protein LNK functions to downregulate JAK/STAT signaling following normal erythropoietin and thrombopoietin stimulation to return cell production to the steady state. Loss of LNK function results in continued erythroid and thrombocytic production.[54] Finally, increased expression of Bcl-$_{XL}$

★ **TABLE 24-15** Classification of Polycythemia

Classification	Associated Conditions
Polycythemia vera (primary)	Normal or decreased erythropoietin levels; autonomous cell proliferation
Secondary polycythemia (erythropoietin driven)	High altitude
	Chronic obstructive pulmonary disease
	Obesity (Pickwickian syndrome)
	Inappropriate erythropoietin production
	Tumors (e.g., hepatoma, uterine fibroma, renal carcinoma)
	Renal ischemia
	Familial erythrocytosis
	Hemoglobins with high oxygen affinity
	Congenital decrease in erythrocyte 2,3-BPG
Relative polycythemia	Gaisböck's syndrome (stress polycythemia, spurious polycythemia, pseudopolycythemia)
	Dehydration

★ **TABLE 24-16** Possible Mechanisms for Increased Erythropoiesis in Polycythemia Vera

- Erythropoietin-independent proliferation of neoplastic progenitor cells
- Hypersensitivity of erythroid progenitor cells to erythropoietin
- Hypersensitivity of erythroid progenitor cells to growth factors other than erythropoietin
- Inhibition of apoptosis in progenitor cells

leads to inhibition of apoptosis in progenitor cells[43] (Chapter 2). The defect in programmed cell death creates an accumulation of altered, hypersensitive stem and progenitor cells. Thrombopoietin receptor hyperresponsiveness and resistance to apoptosis are also found in the megakaryocytic lineage, resulting in the common finding of thrombocytosis associated with PV.[6,43]

Epigenetics

Epigenetic changes contribute to the malignant transformation of PV. In addition to the *TET2* mutation described earlier, somatic mutations in the gene for the isocitrate dehydrogenase (IDH) 1 enzyme that functions in the citric acid cycle manifest epigenetic modifications. IDH1 normally functions to convert isocitrate to α-ketoglutarate with the associated production of NADPH. Mutations have also been identified in IDH2 in patients with MPNs. Epigenetic analysis of IDH1/2 mutations in AML patients has demonstrated hypermethylation. Mutations in these two enzymes occur in PV, ET, and PMF at a rate of 1–5% and are much more frequent (26%) in the later stages of an MPN and after transformation to acute leukemia.[53] It has been speculated that mutational analysis of IDH1 and IDH2 might be useful as a marker for disease progression in patients with MPNs.

Genes and Presentation of Phenotype

Because the *JAK2*(V617F) mutation is found in nearly all patients with PV and in about half of the patients with ET and PMF, it is unclear as to how one mutation can result in three different conditions. It is possible that a dosage effect of the *JAK2* mutation may play an important role in determining disease phenotype since most patients with ET are heterozygous for *JAK2*, whereas PV patients tend to be homozygous. Given the suspicion of a pre-*JAK2* mutation in the MPNs and the discovery of mutated *MPL*, the following mutational sequence has been proposed[54]:

- A pre-*JAK2* mutation produces a hyperproliferative clone with increased susceptibility to additional mutations. Either the *MPL* or *JAK2* mutation occurs, triggering the ET phenotype. The *JAK2* mutation may produce preferential triggering of the MPL receptor over the EPO receptor because MPL receptors have a higher density on the HSC surface.
- When a second *JAK2* mutation occurs, sufficient JAK2 proteins exist to trigger the EPO receptors converting the disease phenotype to PV.
- Additional mutations evolve the phenotype to that of PMF, but thrombocytosis remains because of chemokines released in response to the fibrosis.

Clinical Findings

The annual incidence of PV varies geographically from 2 cases per million in Japan to 13 per million in Australia and Europe. The annual incidence in the United States averages 8–10 per million and occurs most often between the ages of 40–60 years with a peak incidence in the sixth decade of life. The disease is rare in children. It occurs more frequently in males than females and is more common in whites than blacks, particularly in those of Jewish descent.[40] PV has been reported to occur in several members of the same family, suggesting a familial predisposition may exist.

The disease onset is usually gradual with a history of mild symptoms for several years. In some cases, PV is found in asymptomatic individuals. When symptoms are present, they are typically related to the increased erythrocyte mass and the associated cardiovascular disease because of the hyperviscosity of the blood. Headache, weakness, pruritus, weight loss, and fatigue are the most common symptoms. Pruritus is attributed to hyperhistaminemia that can be spontaneous or induced by hot showers or baths. Itching is generalized with absence of a rash.

About one-third of the patients experience thrombotic or hemorrhagic episodes. Myocardial infarctions, retinal vein thrombosis, thrombophlebitis, and cerebral ischemia can occur at any stage of the disease and occasionally can be the first indication of the disease.

When the hematocrit exceeds 60%, the blood viscosity increases steeply, decreasing blood flow and increasing peripheral vascular resistance. These interactions produce hypertension in about 50% of the patients with PV. **Plethora** (a florid complexion resulting from an excessive amount of blood), especially on the face but also on the hands, feet, and ears, is a common finding on physical examination.

After 2–10 years, the patient may develop bone marrow failure accompanied by clinical findings that include an increase in splenomegaly, anemia, and bleeding. Laboratory results are likely to reveal a decreased platelet count and decreasing hematocrit. Together, these findings are known as the **spent phase** and often herald the transition to AML. Postpolycythemic myelofibrosis develops in about 30% of PV cases and splenomegaly is a characteristic finding at the spent phase. Acute leukemia develops as an abrupt transition in 5–10% of patients. Leukemia appears to develop at a higher rate in patients treated with myelosuppressive drugs than in those treated with phlebotomy alone. Overall median survival exceeds 10–20 years.[56]

Laboratory Findings

Peripheral Blood

The most striking peripheral blood finding in PV is an absolute erythrocytosis in the range of $6–10 \times 10^{12}$/L, with a hemoglobin concentration >18.5 g/dL in males and >16.5 g/dL in females. The hematocrit in females is usually >0.48 L/L (48%) and in males is >0.52 L/L (52%). The total RCM is increased >25% of mean normal; the plasma volume can be normal, elevated, or decreased. Early in the disease, the erythrocytes are normocytic, normochromic; however, after repeated therapeutic phlebotomy, iron-deficient erythropoiesis can result in microcytic hypochromic cells. Patients with PV occasionally present with iron deficiency secondary to occult blood loss resulting from abnormal platelet function. This can create a confusing peripheral blood picture because the concentration of erythrocytes is normal to increased with significant microcytosis, simulating a thalassemia (Chapter 14). Nucleated erythrocytes can be found. On the blood smear, the erythrocytes typically appear crowded even at the feathered edge. The reticulocyte count is normal or slightly elevated. The erythrocyte sedimentation rate (ESR) does not exceed 2–3 mm/hour.

Leukocytosis in the range of 12–20 × 10^9/L occurs in about two-thirds of the cases because of an increase in granulocyte production. Early in the disease, there can be a relative granulocytosis and a relative lymphopenia with a normal total leukocyte count. A shift to the left can be found with the presence of myelocytes and metamyelocytes, but finding promyelocytes, blasts, or excessive numbers of immature myeloid cells is unusual. Relative and absolute basophilia is common. The LAP score is usually higher than 100.

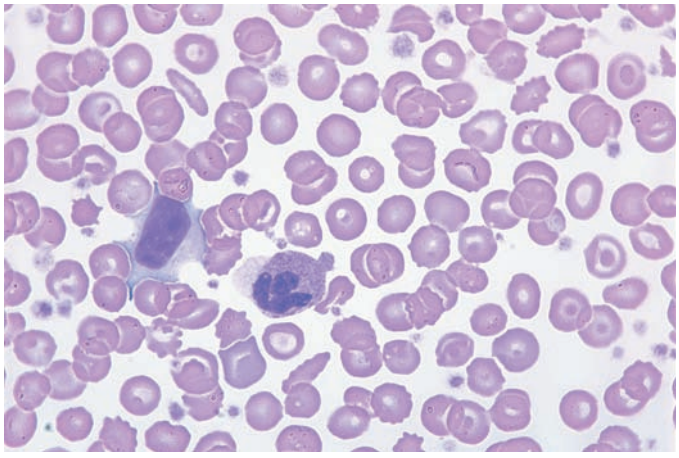
Megakaryocytic hyperplasia in the bone marrow accompanied by an increase in platelet production is a consistent finding in PV. In some patients, the megakaryocytes proliferate without overexpression of the receptor for thrombopoietin (those patients with a mutation in the *MPL* gene) and have decreased apoptosis as do the erythroid progenitor cells.[40,43] The platelet count is >400 × 10^9/L in 20% of PV patients and occasionally exceeds 1000 × 10^9/L. Giant platelets can be found on the blood smear. Qualitative platelet abnormalities are reflected by abnormal aggregation to one or more aggregating agents—epinephrine, collagen, adenosine diphosphate (ADP), or thrombin (Chapters 33, 36). Lack of aggregation with epinephrine is the most common abnormality. The PT and APTT are usually normal (Chapters 34, 36). Abnormal multimeric forms of von Willebrand factor (VWF) are found in about half of PV patients and can lead to a diagnosis of acquired VWD[42] (Chapter 34).

Advanced disease is accompanied by striking morphologic changes in erythrocytes (Figure 24-12 ■). The peripheral blood picture can resemble that of myelofibrosis with a leukoerythroblastic anemia, poikilocytosis with dacryocytes, and thrombocytopenia. In cases that advance to acute leukemia, the blood picture exhibits anemia with marked erythrocyte abnormalities, thrombocytopenia, and blast cells.

Bone Marrow

Most patients with PV have a moderate to marked increase in bone marrow cellularity. The hypercellularity is greater than is seen in secondary polycythemia, and hematopoietic marrow can extend into the long bones. Granulopoiesis as well as erythropoiesis is often increased; consequently, the M:E ratio is usually normal. Although the relative number of myeloblasts is not increased, one of the most significant findings is an increase in megakaryocytes. Eosinophils are also often increased. Sometimes bone marrow biopsies reveal a slight to marked increase in fibrotic material or reticulin, but it is generally directly proportional to the degree of cellularity (e.g., more cellular marrows demonstrating more reticulin). Iron stores are usually absent, presumably because of a diversion of iron from storage sites to the large numbers of developing erythroblasts.

In the postpolycythemic stage, the bone marrow reveals reticulin and collagen fibrosis. Cellularity varies but is often hypocellular with prominent clusters of megakaryocytes. Erythropoiesis and granulopoiesis decrease. A shift to the left can be present, but blasts are usually <10%, and dysplasia is unusual.



■ **FIGURE 24-12** Peripheral blood from a patient with PV showing thrombocytosis and large platelets. A reactive lymphocyte and segmented neutrophil are in the center (Wright-Giemsa stain, 600× original magnification).

Other Laboratory Findings

EPO levels are normal or low in PV, and arterial oxygen saturation levels are normal. In secondary polycythemia resulting from tissue hypoxia, EPO levels are elevated, and arterial oxygen saturation levels are decreased. When secondary polycythemia occurs from an inappropriate increase in EPO, oxygen saturation levels are usually normal.

Other laboratory tests also can be abnormal. Serum uric acid is >7 mg/dL in two-thirds of the patients and can cause symptoms of gout. The increase probably reflects an increase in the turnover of nucleic acids from the increased number of blood cells. Serum cobalamin-binding capacity in most of the untreated PV patients is increased, primarily because of the increase in haptocorrins derived from granulocytes. Serum cobalamin also is increased but not in proportion to the unsaturated binding capacity.

Molecular Genetics

Because of the high frequency of the *JAK2*(V617F) mutation in PV, peripheral blood screening for *JAK2*(V617F) in the initial evaluation of patients suspected of having PV has been recommended. Cytogenetic abnormalities including chromosomal aneuploidy and partial deletions can be found. The most consistent abnormality is a trisomy 8 or 9, an abnormally long chromosome 1, and partial deletions of chromosome 13 and 20. The frequency of multiple karyotypic abnormalities increases after years of treatment and occurs in >80% of patients who develop acute leukemia. Thus, progression from a normal to an abnormal karyotype is an adverse prognostic indicator.

Prognosis and Therapy

There is no known cure for PV, but treatment usually prolongs survival. Without treatment, 50% of the patients survive about 18 months. With only phlebotomy as the palliative treatment, survival extends to about 14 years. Thrombosis is the most frequent complication, and often patients are given antiplatelet therapy.[44]

Phlebotomy

Two types of therapy, phlebotomy and myelosuppressive therapy, have been used historically. Therapeutic phlebotomy is performed to keep the hematocrit below 0.45 L/L (45%) in men and 0.42 L/L (42%) in women and to intentionally reduce iron supplies. Lack of iron is expected to slow the production of erythrocytes, reducing erythrocytosis and the accompanying symptoms.

Chemotherapy

Myelosuppressive or cytoreductive therapy utilizing hydroxyurea, alkylators, interferon, radioactive phosphorus (^{32}P), and anagrelide have been used to reduce the amount of proliferating hematopoietic cells and for patients at risk for vascular events.[40] Hydroxyurea inhibits ribonucleotide reductase and carries less risk of secondary leukemia than does busulphan, an alkylating agent. Younger patients who are intolerant or resistant to hydroxyurea can be treated with α-interferon; busulfan is used for older patients.[57] Low-dose pegylated interferon α-2a has been shown to normalize blood counts in patients with PV and ET, and on sustained therapy, a 30–40% chance that patients could develop a significant molecular response and a significant decrease in the *JAK2(*V617F) allele burden. Pegylated interferon α-2a can retard the progression of ET and PV toward PMF.[58] Patients receiving ^{32}P therapy have a mean survival of 12 years, yet these patients show a progressive incidence of malignant complications.

Molecular Targets

Research to find a molecularly targeted therapy specific for the abnormal JAK2 kinase similar to the use of imatinib in CML is ongoing. The *JAK2* inhibitor, INCB018424 (ruxolitinib), showed promising results in clinical trials. In PV patients, 97% achieved phlebotomy independence and many showed complete remission.[59,60] CEP-701 (lestauritinib, a JAK inhibitor) therapy also revealed a decreased need for phlebotomy and a reduction in *JAK2*(V617F) allele burden.[61]

Differential Diagnosis

It is essential that PV be differentiated from the more benign causes of secondary erythrocytosis and relative polycythemia so that effective therapy can be initiated.

Secondary Polycythemia

Secondary polycythemia can be classified into the following groups:

1. Polycythemia caused by an increase in EPO as a normal physiologic response to tissue hypoxia
2. Polycythemia caused by an inappropriate, nonphysiologic increase in erythropoietin production
3. Familial polycythemia associated with high oxygen-affinity hemoglobin variants
4. Neonatal polycythemia associated with intrauterine hypoxia or late cord clamping

Tissue Hypoxia

A decreased arterial oxygen saturation and subsequent tissue hypoxia are the most common cause of secondary polycythemia. The polycythemia disappears when the underlying cause is identified and effectively treated. Residents of high-altitude areas demonstrate a significant increase in hemoglobin and hematocrit that is progressively elevated at high altitudes. The decrease in barometric pressure at high altitudes decreases the inspired oxygen tension. As a result, less oxygen enters the erythrocytes in the pulmonary alveoli, and the arterial blood oxygen saturation decreases (Chapters 6, 11). Chronic hyperventilation partially compensates for the reduced pO_2 in the lungs. Compensation at the cellular level involves an increase in 2,3-BPG, facilitating the transfer of oxygen to the tissues. Tissue hypoxia secondary to a decrease in arterial blood oxygen saturation can also occur in severe obstructive lung disease and in obesity. The hematocrit is generally not higher than 0.57 L/L (57%) in these cases.

Inappropriate Increase in Erythropoietin

A nonphysiologic increase in EPO (inappropriate) has been described in association with certain tumors that appear to secrete EPO or an EPO-like substance. About 50% of patients who experience this have renal tumors. Other tumors that have been associated with erythrocytosis include those of the liver, cerebellum, uterus, adrenals, ovaries, lung, and thymus. In almost all cases, EPO levels return to normal and the erythrocytosis disappears after resection of the tumor. Renal cysts are also associated with polycythemia, possibly because of localized pressure and hypoxia to the juxtaglomerular apparatus, resulting in increased EPO secretion. In some patients with hypertension, renal artery disease, and renal transplants, renal ischemia can occur, resulting in erythrocytosis secondary to increased EPO production.

Familial Polycythemia

Inherited hemoglobin variants with increased oxygen affinity cause tissue hypoxia and are associated with a secondary erythrocytosis. Because of the increased oxygen affinity, less oxygen is released to the tissues, stimulating erythropoietin production. Inherited deficiency of 2,3-BPG also results in decreased oxygen release to tissues. These inherited conditions are usually found in young children and in other family members as well.

Neonatal Polycythemia

In neonates, hematocrits >0.48 L/L (48%) are common. The etiology is attributed to placental transfusion that occurs as a result of late cord clamping (7–10 seconds after delivery) and/or increased erythropoiesis stimulated by intrauterine hypoxia.[40]

☑ CHECKPOINT 24-7

Renal tumors can produce an inappropriate amount of EPO, resulting in what type of polycythemia?

Relative Polycythemia

Relative polycythemia is a mild polycythemia resulting from dehydration, hemoconcentration, or a condition known as *Gaisböck's syndrome*, which has several synonyms, including *spurious polycythemia*, *pseudopolycythemia*, and *stress erythrocytosis*. Patients with these conditions have a relative polycythemia and hypertension with nephropathy or relative polycythemia associated with emotional stress.[43] RCM is essentially normal. High hematocrit and hemoglobin concentrations appear to result from a combination of high-normal erythrocyte concentrations with a low-normal plasma volume. The most common symptoms are light-headedness, headaches, and

dizziness. Plethora is common but splenomegaly is rare. These patients have a high incidence of thromboembolic complications and cardiovascular disease. Although the hemoglobin, hematocrit, and erythrocyte counts are increased, leukocytes and platelets are normal. Bone marrow cellularity is normal with no increase in megakaryocytes or reticulin. Bone marrow iron stores are absent in 50% of the patients, but serum iron studies are normal. Chromosome karyotypes are almost always normal.

Laboratory Differentiation of Polycythemia

The WHO-defined diagnostic criteria for PV include initial determination of total RCM or hemoglobin to establish the presence of an absolute polycythemia[62] (Table 24-17 ★). However, it has been shown that in some cases with hemoglobin levels below the value used for a diagnosis of PV and thrombocytosis, the RCM was higher than that required, thus revealing an occult erythrocytosis.[65] Without the RCM, these cases would have been diagnosed as essential thrombocythemia. This suggests that RCM is an important test to perform when PV is suspected. Determination of serum EPO levels is important because it helps to distinguish between primary and secondary polycythemia. If EPO levels are low, screening for the *JAK2*(V617F) mutation and bone marrow histology should be performed.[40] An elevated EPO indicates secondary polycythemia.

☑ CHECKPOINT 24-8

Which of these conditions—iron deficiency, smoking, emphysema, pregnancy, dehydration—are associated with an absolute increase in RCM?

★ **TABLE 24-17** Polycythemia Vera Diagnostic Criteria[62,63,64]

PV diagnosis requires major criteria (1) and (2) and one minor criterion or major criterion (1) and two minor criteria

Major criteria	1. Hb >18.5 g/dL in men or >16.5 g/dL in women or >99th percentile of method-specific reference interval[a] 2. Presence of *JAK2*(V617) mutation or other similar mutation such as *JAK2* exon 12 mutation
Minor criteria	1. Bone marrow biopsy shows panmyelosis with proliferation of myeloid elements (erythroid, myeloid, and megakaryocytic cells) 2. Decreased serum erythropoietin (below the reference interval or <4 mU/mL) 3. Demonstration of endogenous erythroid colony formation *in vitro*

[a]If there is an increase of ≥2 g/dL from the patient's baseline level, then a hemoglobin >17 g/dL in men or >15 g/dL in women.

Erythropoietin Measurement

EPO is critical in differentiating PV from secondary polycythemias. With the *JAK2*(V617F) mutation, erythropoiesis occurs without the need for EPO stimulation. Serum EPO levels are usually very low or not detectable in PV. Secondary causes of polycythemia are related to elevated EPO levels either because of hypoxia or an inappropriate release of EPO from the kidneys (tumors and renal carcinomas).

Genetic Studies

Studies should include molecular analysis for the *JAK2*(V617F) mutation found in exon 14. In addition, 10 different mutations have been identified in exon 12 of *JAK2* that are associated with erythrocytosis. Karyotype screening for trisomies of chromosome 8 and 9 as well as deletions of 13 or 20 may also be performed.[40]

Bone Marrow Changes

A bone marrow assessment can be helpful in patients in whom EPO is not low and *JAK2*(V617F) is not detected, but an elevated RCM and clinical symptoms suggest PV. Histologic changes for PV include hypercellularity with increased erythroid precursors, increased granulocytic and megakaryocytic cells with megakaryocyte clusters, and reticulin fibrosis.[40]

In any classification scheme, recognizing the possibility of two coexisting disease states is imperative. For instance, a patient can have both PV and a secondary polycythemia as occurs in chronic obstructive pulmonary disease. Refer to Table 24-18 ★ for differentiating features of polycythemia vera from secondary and relative polycythemia.

★ **TABLE 24-18** Differential Features of Polycythemia

Feature	PV	Secondary	Relative
Spleen size	↑	N	N
RCM	≥36 mL/kg (males) ≥32 mL/kg (females)	↑	N
Leukocyte count	↑	N	N
Platelet count	↑	N	N
Serum cobalamin	↑	N	N
Arterial O_2 saturation	N	↓	N
Bone marrow	Panhyperplasia, reticulin deposits	Erythroid hyperplasia	N
LAP	N to ↑	N	N
Iron stores	↓	N	N
EPO	N, ↓	N, ↑	N
Chromosome studies	Abnormal, >90% *JAK2*(V617F)+	N	N

N = normal; ↑ = increased; ↓ = decreased; LAP = leukocyte alkaline phosphatase; EPO = erythropoietin.

PRIMARY MYELOFIBROSIS (PMF)

Primary myelofibrosis (PMF) is a clonal hematopoietic stem cell disorder with splenomegaly, leukoerythroblastosis, extramedullary hematopoiesis (myeloid metaplasia), and progressive bone marrow fibrosis.[66] The proliferation of hematopoietic cells in the early stages of the disease is neoplastic and presents either as a de novo condition or as an evolutionary consequence of PV or ET. The fibroblast (collagen-producing cell) is an important component of normal bone marrow in which the fibroblasts provide a support structure for hematopoietic cells. Fibroblast proliferation in PMF is reactive and secondary to the underlying disorder. Fibrotic tissue eventually disrupts the normal architecture and replaces hematopoietic tissue in the bone marrow. Excessive marrow fibrosis inhibits normal hematopoiesis, producing marrow hypoplasia and stimulates myeloid metaplasia. Myeloid metaplasia usually occurs in both the spleen and the liver. These organs can become massive in size as the result of islands of proliferating erythroid, myeloid, and megakaryocytic elements. The extramedullary hematopoiesis is similar to that occurring during embryonic hematopoiesis.

PMF has been known by many synonyms. Most of the different names were attempts to describe the typical blood, bone marrow, and spleen abnormalities. Some of the terms that have been used include *agnogenic myeloid metaplasia*, *myelofibrosis with myeloid metaplasia*, *chronic idiopathic myelofibrosis*, *aleukemic myelosis*, *myelosclerosis*, *splenomegalic myelophthisis*, and *leukoerythroblastic anemia*.

Etiology and Pathophysiology

PMF is associated with a profound hyperplasia of morphologically abnormal megakaryocytes (dysplastic and necrotic). In most cases, only megakaryocytes and granulocytes are involved, but all three lineages, including erythrocytes, can be involved in the disease process. PMF is often preceded by a hypercellular phase of variable duration. The disease evolves from this prefibrotic stage with minimal reticulin fibrosis to a fibrotic stage with marked reticulin or collagen fibrosis. Thus, at diagnosis, the bone marrow can exhibit varying degrees of fibrosis. The fibrosis is not considered part of the primary abnormal clonal proliferation but is a secondary reactive event occurring in response to the progeny of the clonal hematopoietic cells and, in fact, fibroblasts do not contain the chromosome abnormalities found in the hematopoietic cells.

Understanding of this disease has increased considerably with a better understanding of normal bone marrow structure and the changes that occur in myelofibrotic marrow associated with megakaryocyte growth factors that mediate fibrogenesis.[67] The bone marrow extracellular matrix or microenvironment (stroma) supports hematopoietic cell proliferation (Chapter 3). Myelofibrotic stroma is characterized by an increase in total collagen, fibroblasts, vitronectin (a cytoadhesion molecule), fibronectin (a cytoadhesion molecule normally limited to megakaryocytes and walls of blood vessels), and laminin (a glycoprotein that supports adhesion and growth of cells).

Cytokine Involvement

Megakaryocytes play an important role in the pathogenic development of the abnormal PMF marrow. In areas of megakaryocyte necrosis, fibroblast proliferation and collagen deposition often are prominent. This stromal reaction is a cytokine-mediated process.[67] PDGF, epidermal growth factor (EGF), and transforming growth factor beta (TGF-β) are contained in the α-granules of megakaryocytes and platelets, and all stimulate the growth and proliferation of fibroblasts. Reduced platelet concentrations of PDGF and increased levels of serum PDGF are characteristic of PMF. This condition is thought to represent the abnormal release or leakage of the growth factor from the platelet. PDGF does not stimulate synthesis of collagen, laminin, or fibronectin, but TGF-β stimulates increased expression of genes for fibronectin and collagen while it decreases synthesis of collagenase-like enzymes. Thus, the net effect is the accumulation of bone marrow stromal elements.[67]

Gene Mutations

About 50% patients can have a somatic mutation in either the *JAK2* or *MPL* genes in a hematopoietic stem cell. The proto-oncogene *CBL* is mutated primarily in juvenile myelomomocytic leukemia (JMML) or chronic myelomonocytic leukemia (CMML) and has been identified in 6% of patients with PMF.[68] Chromosome aberrations, when present, are restricted to cells derived from the mutated HSCs. Some of these cells are highly sensitive to or independent from regulation by their respective stimulatory factors. The disorder has been reported to terminate in ALL as well as AML in some patients.

JAK2(V617F) is found in approximately 50% of patients with PMF, and the *JAK2*(V617F) allele burden has been associated with disease progression based on several observations. First, *JAK2* homozygosity is identified more often in PV and PMF than in ET. Second, increased *JAK2* allele burden has been associated with increased symptoms to include cardiovascular events. Third, *JAK2* allele burden might increase the risk of transformation into PMF. In contrast, PMF patients negative for *JAK2* and with lower *JAK2* burden experience shorter survivals.[69–73]

☑ CHECKPOINT 24-9

What growth factors are primarily responsible for stimulating fibrogenesis in the bone marrow?

Clinical Findings

PMF generally affects individuals older than 50 years of age and seems to occur equally between sexes. It rarely occurs in childhood. Its onset is gradual, and the disease is chronic. Early in the disease process, the patient might not have symptoms, making the time of onset difficult to determine. If symptoms are present, they are usually related to anemia or pressure from an enlarged spleen. Bleeding occasionally is a presenting symptom. Patients complain of weakness, weight loss, loss of appetite, night sweats, pruritus, pain in the extremities, bone pain, discomfort in the upper left quadrant, and fever. The major physical findings are splenomegaly (in 90%),

hepatomegaly (in 50%), pallor, and petechiae.[74,75] Myeloid metaplasia is found in the spleen and frequently in the liver and can be found in the kidney, adrenal glands, peritoneal and extraperitoneal surfaces, skin, lymph nodes, and spinal cord. Osteosclerosis is a frequent finding and, when found in association with splenomegaly, suggests a diagnosis of myelofibrosis.

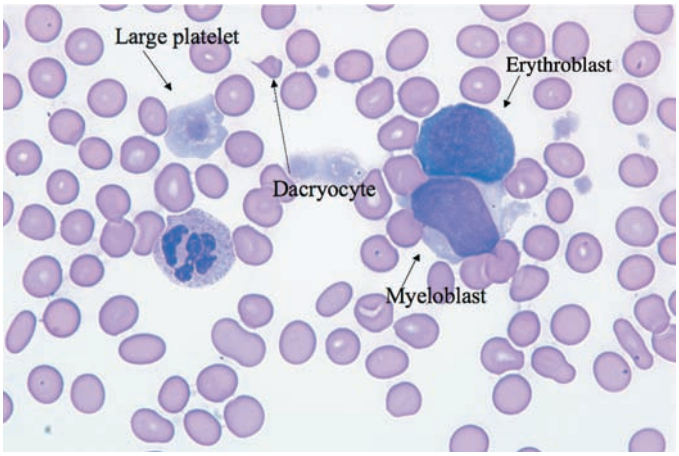
An atypical acute form of the disease has been described with a rapid and progressive course of a few months to 1 year. Anemia develops rapidly, and the leukocyte count is decreased. The bone marrow in these cases exhibits a proliferation of reticular and collagen fibers.

Patients with systemic lupus erythrematosus (SLE) can present with myelofibrosis that is morphologically indistinguishable from the myelofibrosis of PMF. These patients also have various peripheral blood cytopenias similar to those found in PMF but not splenomegaly. The myelofibrosis in SLE has been referred to as *autoimmune myelofibrosis*. It has been suggested that all patients with myelofibrosis and an absence of splenomegaly should have an antinuclear antibody (ANA) test to rule out SLE.[76]

Laboratory Findings

Peripheral Blood

The typical peripheral blood findings for PMF reflect both qualitative and quantitative cellular abnormalities. At diagnosis, there may be anemia, leukocytosis with a left shift, and thrombocytosis. Pancytopenia and leukoerythroblastosis with striking anisocytosis and poikilocytosis is typical at later stages of the disease (Figure 24-13 ■). The anemia is usually normocytic, normochromic, but hypochromia can be found if the individual has a history of hemorrhage or hemolysis. Between 35–54% of patients with PMF have a hemoglobin level of <10 g/dL.[77] Folic acid deficiency can develop as a result of increased utilization by the neoplastic clone and is associated with a macrocytic anemia. Anemia uncomplicated by iron deficiency or folic acid deficiency correlates directly with the extent of bone marrow fibrosis and the effectiveness of extramedullary hematopoiesis. The anemia becomes more severe with the progression of the disease and is aggravated in some patients by the combination of splenomegaly, which causes sequestration of erythrocytes, and expanded plasma volume (dilutional anemia).



■ **FIGURE 24-13** Peripheral blood from a patient with PMF. Leukoerythroblastic picture with an erythroblast and a myeloblast below it. The arrow points to a large platelet. Other abnormal platelets are present. Note the poikilocytosis with dacryocytes (Wright-Giemsa stain, 1000× magnification).

The presence of abnormal erythrocyte morphology is an important feature of PMF. The most typical poikilocyte is the dacryocyte, although elliptocytes and ovalocytes are also present (Figure 24-13). A few nucleated erythrocytes are usually found and sometimes can be numerous. Basophilic stippling is a common finding. Reticulocytosis is typical, ranging from 2–15%. The majority of patients have an absolute reticulocyte $> 60 \times 10^9$/L.

The leukocyte count is usually elevated but can be normal or, less often, decreased upon initial presentation. The count generally ranges from 15 to 30 $\times$ 10^9/L. A leukocyte count above 60–70 $\times$ 10^9/L prior to splenectomy is rare. As the disease progresses, the leukocyte concentration does not decrease as quickly as the erythrocytes and platelets. An orderly progression of immature granulocytes is characteristically found. Blasts generally compose $<5\%$ of circulating leukocytes. Other common findings include basophilia, eosinophilia, and pseudo–Pelger-Huët anomaly. The LAP is elevated or normal but occasionally is decreased. Low LAP scores correlate with leukopenia. When elevated, the LAP score helps to differentiate this disease from CML. The Ph chromosome is not present.

Platelets can be decreased, normal, or increased. Higher counts are associated with early disease stages; thrombocytopenia is usually found in the later stages. Thrombocytopenia is often attributed to excessive splenic pooling. The platelets can appear dysplastic: typically giant, bizarre, and frequently hypogranular. Circulating megakaryocyte fragments, mononuclear micromegakaryocytes, and naked megakaryocyte nuclei can exist. The micromegakaryocytes can present an identification problem because they frequently resemble lymphocytes. However, important differentiating features of micromegakaryocytes are the presence of demarcation membranes with bull's-eye granules in the cytoplasm and cytoplasmic blebbing. Qualitative platelet abnormalities including abnormal aggregation, adhesiveness, and defective platelet procoagulant activity on exposure to collagen are consistent findings.

Among patients with PMF, 15% have major hemolytic episodes during the course of their disease.[66] Hemosiderinuria and decreased haptoglobin are found in about 10% of patients, suggesting intravascular hemolysis. The cause of hemolysis can be hypersplenism, PNH-like defective erythrocytes, and antierythrocyte antibodies.

A bleeding diathesis ranging from petechiae and ecchymoses to life-threatening hemorrhage can be found in some patients, likely resulting from a combination of thrombocytopenia and/or abnormal platelet function. Defective platelet aggregation is a common finding. Hemostatic abnormalities suggestive of chronic DIC, including decreased platelet count, decreased concentration of factors V and VIII, and increased fibrin degradation products, can be present.[66]

Other laboratory tests for PMF are frequently abnormal. Serum uric acid and LD are elevated in most patients. Serum cobalamin can be slightly increased but is usually normal.

☑ CHECKPOINT 24-10

What erythrocyte morphologic feature is a hallmark for myelofibrosis?

Bone Marrow

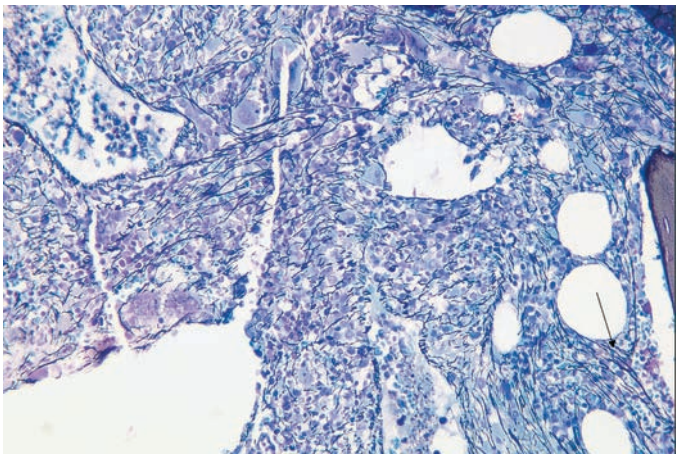
The bone marrow is difficult to penetrate and frequently yields a dry tap. If aspiration is successful, smears may show no abnormalities; biopsy specimens are needed to reveal the extent of fibrosis. In most PMF cases, the marrow is hypercellular with varying degrees of diffuse fibrosis and focal aggregates of megakaryocytes (Figure 24-14 ■).

Three bone marrow histologic patterns have been described: (1) panhyperplasia with absence of myelofibrosis but a slight increase in connective tissue reticulin, (2) myeloid atrophy with fibrosis, prominent collagen and reticulin fibers, and cellularity <30%, and (3) myelofibrosis and myelosclerosis with bony trabeculae occupying 30% of the biopsy and extensive fibrosis.

CASE STUDY *(continued from page 454)*

During the office visit, Roger stated his symptoms of fatigue, weakness, dyspnea, bone pain, and abdominal discomfort. A bone marrow biopsy was ordered. The aspiration was unsuccessful, but the marrow biopsy showed moderate to marked hyperplasia, clusters of platelets, abnormal megakaryocyte morphology, and fibrotic marrow spaces.

4. What diagnoses do these results suggest?
5. Give a reason for the unsuccessful, dry-tap bone marrow aspiration.
6. What characteristic peripheral blood morphologies correlate with the bone marrow picture and physical exam?



■ **FIGURE 24-14** Bone marrow stained with reticulin stain shows increased collagen (black-staining fibers) in a patient with PMF (BM biopsy, reticulin stain, 100× magnification).

Molecular Genetics

Cytogenetic analysis is important to differentiate myelofibrosis from other myeloproliferative disorders, particularly CML, which also can have some degree of fibrosis. The Ph chromosome is not present in PMF. Although no specific cytogenetic abnormality is diagnostic for PMF, *JAK2*(V617F) is found in about 50% of patients, and a trisomy or deletion of group C chromosomes (chromosomes 6–12) is also associated with myelofibrosis. Complete or partial loss of chromosomes 5, 7, and 20 is associated with PMF patients treated with chemotherapy.

Prognosis and Therapy

The average survival time after diagnosis is 4–5 years. The main causes of death are infection, hemorrhage, thrombosis, and cardiac failure. About 10–15% of patients progress to an acute myelogenous leukemia and some to acute lymphoblastic leukemia.

Therapy is selected to achieve two particular outcomes: improve cytopenia and reduce massive splenomegaly. Corticosteroids, androgens, and recombinant erythropoietin are used to stimulate erythropoiesis to reduce anemia, but patients may still require periodic transfusions.[67] When anemia cannot be controlled, splenectomy can be considered.

Irradiation has been suggested to decrease spleen size in an attempt to relieve symptoms or to decrease excessive erythrocyte destruction. Hydroxyurea has been used, but it can cause severe pancytopenia. Thalidomide and lenalidomide also have been used to treat splenomegaly as well as chemotherapeutic agents such as 2-chlorodeoxyadenosine (2-CdA or Leustatin) when the splenomegaly is advanced.[67] Neuropathy has been reported with thalidomide, and myelosuppression can occur with lenalidomide. A similar agent, pomalidomide, has produced anemia and symptom relief without neuropathy and myelosuppression.[78] Allogeneic stem cell transplantation is the only curative therapy for PMF, but the advanced age of many patients is a factor in the high mortality rate.

JAK inhibitors are being investigated with varying success. Ruxolitinib, a JAK1/2 inhibitor that when administered to patients with advanced PMF at maximum tolerated doses daily, resulted in clinical improvement for some patients.[79] However, ruxolitinib did not impact *JAK2*(V617F) allele burden. CYT387, another JAK1/2 inhibitor, is associated with splenic reduction and some degree of symptom relief.[36] Clinical trials with drugs that are JAK2/FLT3 inhibitors have variable clinical effects.[57]

Differential Diagnosis

Differentiating PMF from other conditions associated with fibrosis is essential to ensure appropriate therapeutic regimens (Table 24-19 ★). Splenomegaly, anemia, and a leukoerythroblastic blood picture are significant findings in both myelofibrosis and CML. In myelofibrosis, the leukocyte count is generally $<50 \times 10^9$/L, whereas in CML, the count is expected to be higher. In myelofibrosis, the granulocyte left shift is less pronounced and poikilocytosis is striking. The bone marrow in myelofibrosis is fibrous with large numbers of megakaryocytes. In CML, the bone marrow can also exhibit some fibrosis, but the most abnormal finding is the myeloid hyperplasia. The serum cobalamin level is not as elevated in myelofibrosis as it is in CML. The LAP score

★ **TABLE 24-19** Conditions Associated with Marrow Fibrosis

Neoplasms	Other
Primary myelofibrosis	Miliary tuberculosis
Chronic myelogenous leukemia	Fungal infection
Polycythemia vera	Granulomas
Essential thrombocythemia	Marrow damage by radiation or chemicals
Megakaryocytic leukemia	
Metastatic carcinoma	
Hairy cell leukemia	
Lymphoma	
Hodgkin's disease	
Acute megakaryocytic leukemia	

in myelofibrosis is variable, but when elevated, it is strong evidence against CML. The most reliable test to differentiate CML and PMF is cytogenetic analysis for the Ph chromosome.

Differentiating PMF from polycythemia vera, especially in the later stages, is more difficult. The later stages of PV can be accompanied by increased marrow fibrosis and actual transformation to PMF can occur. When thrombocytosis is the principal initial hematologic finding, PMF can be confused with ET. A bone marrow biopsy aids in the differentiation, revealing fibrosis in PMF.

CASE STUDY *(continued from page 469)*

Over the next several months, Roger experienced increasing splenomegaly and abdominal discomfort. Cytogenetic studies revealed a trisomy 8.

7. What is the most likely explanation for the increased splenomegaly?
8. What are possible outcomes of this disorder?

MYELOPROLIFERATIVE NEOPLASM, UNCLASSIFIABLE (MPN, U)

Myeloproliferative neoplasm, unclassified (MPN, U) is the diagnosis for cases that have the characteristic clinical, laboratory, and morphologic features of an MPN but do not meet the specific criteria for one of the other MPN categories or have features that overlap two or more categories. The *Ph* chromosome and *BCR/ABL1* fusion gene are absent, and the cell of origin is most likely the pluripotent HSC.

Most cases of MPN, U are either very early stages of PV, PMF, or ET or are at a late stage of advanced MPN in which extensive myelofibrosis, osteosclerosis, or transformation to an aggressive stage obscures the true disorder. Follow-up at intervals can permit an accurate diagnosis. A specific diagnosis should be made as soon as possible because of the therapeutic implications for each of the neoplastic disorders.

The incidence of MPN, U is unknown but can be as high as 20% of MPNs. Clinical features are similar to those found in other MPNs, including splenomegaly and hepatomegaly.

Laboratory Findings

Leukocytes and platelets can be increased. Hemoglobin is variable. The bone marrow is hypercellular and often shows megakaryocytic hyperplasia and variable granulocytic and erythroid proliferation. In advanced stages, the bone marrow is fibrotic or osteomyelosclerotic. If there are 10–19% blasts in the peripheral blood, the disease is diagnosed as an accelerated stage. No specific cytogenetic or molecular abnormalities are identified with this disorder.

CLONAL HYPEREOSINOPHILIA

Eosinophils are derived from the colony-forming units generating granulocytes, erythroblasts, macrophages, and megakaryocytes (CFU-GEMM and CMP) through the action of eosinophilic cytokines, GM-CSF, IL-3, and IL-5. IL-5 is relatively lineage specific for eosinophils (Chapters 4, 7). The cells are released into the peripheral blood and rapidly migrate to tissues where they perform their physiologic function. Diagnosis of disorders associated with eosinophilia may be urgent because of potential damage to organs infiltrated with eosinophils and subsequent release of cytokines, enzymes, and other proteins.

The term **hypereosinophilic syndrome (HES)** describes a group of disorders that demonstrate an absolute eosinophil count of $>1.5 \times 10^9$/L that persists for >4 weeks.[80] The eosinophilia can result from a clonal (neoplastic) disorder or a nonclonal (benign or reactive) disorder or can be of unknown origin (idiopathic-hypereosinophilic syndrome, I-HES) (Table 24-20 ★).

Reactive, nonclonal (benign) hypereosinophilic disorders result from increased production of eosinophil cytokines secondary to or associated with another diagnosis (Chapters 7, 21). Diseases typically associated with benign eosinophilia must be ruled out before considering a neoplastic diagnosis (Table 24-20). See Table 24-21 ★ for tests to exclude diseases associated with reactive (secondary) eosinophilia.[81] It is especially important to perform serological testing to rule out infection with *Strongyloides* sp. because patients with this infection who are given corticosteroids (common treatment for I-HES) can experience dissemination of the disease, which can be fatal.[82] Once it is found that the hypereosinophilic condition is not clonal, testing to identify reactive conditions such as allergies, parasitic infections, and asthma, must be performed. In some cases, no underlying cause for the eosinophilia can be found and clonality cannot be proven. This disorder is known as I-HES (Table 24-20). Many disorders that were previously considered as I-HES are now included in subgroups with specific genetic abnormalities.

Clonal hypereosinophilia is considered a progenitor cell disorder (primary) that includes a new myeloid/lymphoid neoplasm group added by the WHO in 2008 termed myeloid and lymphoid neoplasms with eosinophilia and abnormalities of platelet-derived growth factor receptor α (PDGFRA), PDGFR β (PDGFRB), or fibroblast

★ **TABLE 24-20** Conditions of Hypereosinophilia

Conditions	Examples
Neoplastic conditions (clonal)	Myeloproliferative neoplasms
	• Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
	• Chronic myeloid leukemia (CML)
	• Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)
	• Chronic myelomonocytic leukemia (CMML)
	• Myelodysplastic syndromes
	• Systemic mastocytosis (SM)
	Myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB, or FGFR1
	• Myeloid and lymphoid neoplasms associated with *PDGFRA* rearrangement
	• Myeloid neoplasms associated with *PDGFRB* rearrangement
	• Myeloid and lymphoid neoplasms associated with *FGFR1* abnormalities
	Acute leukemias (AML or ALL)
Benign, reactive conditions (nonclonal)	Parasitic infection
	Asthma
	Allergies
	Skin diseases
	Loeffler's syndrome
	Vasculitis
	Drug hypersensitivity
Idiopathic-hypereosinophilic syndrome	

growth factor receptor 1 (FGFR1)[80] and **chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)**, a subgroup of MPNs (Table 24-20). The eosinophil may also be a part of the neoplastic clone of cells found in other neoplasms such as MPNs, MDS, AML, and ALL. Cytogenetics or molecular analysis can help clarify the origin of eosinophils in these neoplasms.

★ **TABLE 24-21** Suggested Information and Testing to Exclude Diseases Associated with Reactive (secondary) Eosinophilia

Clinical	Patient history
	Physical examination
Laboratory tests	Complete blood count and differential
	Bone marrow aspiration and biopsy
	Routine chemistries
	Serum IgE
	Cobalamin
	HIV serology
	Serology testing and stool analysis for parasites
Other tests	Pulmonary function tests
	Chest and abdominal CT scan

Myeloid and Lymphoid Neoplasms Associated with Eosinophilia and *PDGFRA*, *PDGFRB*, or *FGFR1* Mutations

These are a group of neoplastic eosinophil disorders caused by mutations in genes that encode the α- or β-chains of the protein tyrosine kinases platelet-derived growth factor receptors (*PDGFRA*, *PDGFRB*) or in the fibroblast growth factor receptor 1 (*FGFR1*). These gene mutations result in constitutive activation of intrinsic tyrosine kinase activity (Chapter 4). All three disorders can present as a chronic myeloproliferative disorder. Less frequently, they can present as AML or precursor T lymphoblastic leukemia/lymphoma. Recognition of these disorders is important because the mutations result in abnormal tyrosine kinases that may respond to treatment with imatinib or other TKIs. Eosinophilia is variable but present in most cases.

Myeloid and Lymphoid Neoplasms with *PDGFRA* Rearrangement

A gain of function tyrosine kinase fusion gene was discovered when some HES patients who were unresponsive to corticosteroid therapy were found to respond to imatinib therapy. The gene fusion was found to occur between the *FIP1L1* gene and the *PDGFRA* gene. The mutation results from a small interstitial deletion in chromosome 4, del(4)(q12q12), resulting in the *FIP1L1/PDGFRA* (*F/P*) gene[80] and an abnormal, constitutively activated tyrosine kinase protein. The cell of origin is the pluripotential hematopoietic stem cell. The detection of a fusion gene in a particular lineage does not always correlate with the morphological presentation.

Clinical Findings

Most of the cases involving the *F/P* mutation are found in males (17:1 male-to-female ratio), and age at onset is 25–55 years.[82] There is evidence of eosinophil-related tissue damage and tissue fibrosis. Splenomegaly is present.

Laboratory Findings

The initial laboratory finding is usually hypereosinophilia but can be AML or T lymphoblastic leukemia/lymphoma. Eosinophils are mature with few myelocytes or promyelocytes. Eosinophil granulation may be sparse with vacuoles present. Hyper- or hyposegmentation of the nucleus can be seen. A few patients have an increase in blasts, but the peripheral blood and/or bone marrow contain $<$20% blasts. Anemia and thrombocytopenia may be present. Increased serum tryptase and cobalamin are typical. The bone marrow is hypercellular with increased eosinophil precursors. Charcot-Leyden crystals may be seen. Many cases show an increase in spindle-shaped atypical mast cells with a CD25+ immunophenotype. These findings also are present in a significant number of patients who have systemic mastocytosis with increased atypical (spindle-shaped) mast cells in the bone marrow and a mutation in the stem cell factor receptor *c-Kit*. The *F/P* gene can be demonstrated by RT-PCR or FISH techniques (Chapters 41, 42).

Therapy

Although most patients who harbor the *F/P* mutation respond to imatinib therapy, variations within the F/P mutation have recently been identified as resistant to imatinib. The second generation nilotinib and the third generation ponatinib TKIs have shown promising results in imatinib-resistant patients.[80]

Myeloid Neoplasms with *PDGFRB* Rearrangement

The myeloid neoplasms with *PDGFRB* rearrangement are characterized by a t(5;12) (q33;p13) rearrangement (most commonly) to form the *ETV6-PDGFRB* fusion gene. Other translocations with a 5q31–33 breakpoint can occur and lead to other fusion genes, but they are not included in this subgroup and are not likely to respond to imatinib therapy. The postulated cell of origin is a multipotential HSC that is able to differentiate to neutrophils, monocytes, eosinophils, and probably mast cells.

Clinical Findings

Myeloid neoplasms with *PDGFRB* rearrangement can present as CMML with eosinophilia, aCML, or as MPN with eosinophilia. Acute (blast) transformation can occur. There is a 2:1 male predominance and a wide variability in age (8–72 years) at onset.

Laboratory Findings

The leukocytes are increased with a variable increase in eosinophils, neutrophils, monocytes, and precursor cells. Anemia and thrombocytopenia may be present. The bone marrow is hypercellular with an increase in mast cells that can be spindle shaped. Reticulin can be increased. The peripheral blood and bone marrow have $<$20% blasts. Molecular analysis using primers for all known breakpoints to confirm *ETV6-PDGFRB* is recommended. Cytogenetic analysis usually reveals the t(5;12).

Therapy

Before imatinib therapy, survival with myeloid neoplasms with *PDGFRB* rearrangement was $<$2 years. Patients usually respond to imatinib treatment.[80] Survival is expected to improve with early diagnosis and treatment before organ damage occurs.

Myeloid and Lymphoid Neoplasms with *FGFR1* Abnormalities

The myeloid and lymphoid neoplasms with *FGFR1* abnormalities subgroup of eosinophilic neoplasms are characterized by an 8p11 breakpoint, which, depending on the chromosome partner, results in a variety of fusion genes that incorporate part of the *FGFR1* gene. All fusion genes encode a tyrosine kinase that has constitutive activity. Other cytogenetic abnormalities can occur. The postulated cell of origin is the pluripotential hematopoietic stem cell.

Clinical Findings

There is a male predominance with the myeloid and lymphoid neoplasms in the *FGFR1* abnormalities subgroup (1.5:1), and a wide age range at onset (3–84 years) is observed.

Laboratory Findings

This neoplasm presents as a hypereosinophilia, or if transformed to an acute neoplasm, as an AML or precursor T or B lymphoblastic leukemia/lymphoma or as a mixed phenotype acute leukemia. In patients who are diagnosed in acute transformation, the myeloblasts, lymphoblasts, and eosinophils belong to the neoplastic clone. The chronic phase usually has eosinophilia, neutrophilia, and occasionally monocytosis.

Therapy

Prognosis is poor. Tyrosine kinase inhibitors are not currently effective although second- and third-line TKIs are showing promising results.[80] Bone marrow transplant can be indicated in the chronic phase.

Chronic Eosinophilic Leukemia, Not Otherwise Specified (CEL-NOS)

CEL-NOS is a clonal myeloproliferative neoplasm that presents with eosinophilia not classified as another neoplastic condition and does not have the *PDGFRA*, *PDGFRB*, or *FGFR1* mutations. In addition, reactive eosinophilia, diseases associated with abnormal release of cytokines (IL-2, IL-3, IL-5, or GM-CSF), or presence of aberrant T cells must be ruled out. The diagnostic criteria for CEL-NOS[63,83] are listed in Table 24-22 ★.

The cell of origin for this leukemia may be the hematopoietic stem cell (HSC), multipotential progenitor cell (MPP), or a committed eosinophilic progenitor cell (CFU-Eo). In some cases, cytogenetic abnormalities such as trisomy 8 can be identified.[80]

Clinical Findings

CEL-NOS is most often diagnosed in middle-aged men (male-to-female ratio is 9:1). Presenting symptoms include fever and significant weight loss. Clinical features include central nervous system (CNS) irregularities, hepatosplenomegaly, congestive heart failure, pulmonary fibrosis, and occasionally lymphadenopathy. Release of excessive eosinophilic granules in the blood causes fibrosis of the endothelial cells resulting in peripheral vasculitis, gangrene of digits, and organ damage, particularly of the heart and lungs.[80]

Laboratory Findings

CEL-NOS is characterized by a peripheral blood eosinophilia of 1.5×10^9/L or more. There is evidence of clonality and/or $>$2% blasts in the peripheral blood or 5–19% blasts in the bone marrow. The leukocyte count is usually $>30 \times 10^9$/L with 30–70% eosinophils. Anemia and thrombocytopenia can be present. The LAP score is normal, but the serum cobalamin, uric acid, and muramidase are frequently elevated. The bone marrow shows a left shift with many eosinophilic myelocytes. Marrow fibrosis is a common finding, and Charcot-Leyden crystals are often seen.

Therapy

When therapy is ineffective, the prognosis for patients with CEL-NOS is poor. Few live beyond 1 year after diagnosis. The major cause of death is congestive heart failure from tissue injury.

Differential Diagnosis

Ruling out conditions associated with reactive eosinophilia is important. CEL-NOS also should be differentiated from clonal eosinophilia found in other hematologic disorders (Table 24-20). If there is no evidence of a reactive eosinophilia, clonality, or increase in blasts, the diagnosis should be I-HES.

★ **TABLE 24-22** Diagnostic Criteria for Chronic Eosinophilic Leukemia, Not Otherwise Specified (CEL-NOS)—Persistent Eosinophilia $\geq 1.5 \times 10^9$/L in Blood

- <20% blasts in blood and bone marrow; no inv(16)(p13.1q22) or t(16;16)(p13.1:q22) or other diagnostic feature of AML
- Exclude eosinophilic disorders that bear the *PDGFRA*, *PDGFRB*, or *FGFR1* mutations
- No *BCR/ABL1* fusion gene; absence of other MPNs, MDS/MPN
- Exclude all secondary causes of eosinophilia
- Exclude neoplastic disorders with secondary eosinophilia
- Exclude neoplastic disorders when eosinophils are a part of the neoplastic clone (e.g., AML)
- Exclude presence of an aberrant phenotypic T-cell population
- With all of the preceding conditions and evidence of clonality or >2% blasts in the peripheral blood and >5% blasts in the bone marrow, diagnosis is CEL

If a patient has persistent eosinophilia but does not meet these criteria, the diagnosis can be reactive eosinophilia, idiopathic hypereosinophilia, or idiopathic hypereosinophilic syndrome.

Gotlib J. World Health Organization-defined eosinophilic disorders: 2012 update on diagnosis, risk stratification and management. *Am J Hematol*. 2012;87(9):903–14; Vardiman JW, Thiele J, Arber DA et al. The 2008 revision of the World Health Organization (WHO) classification of the myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–51. Bain BJ, Gilliland DG, Vardiman JW et al. Chronic eosinophilic leukemia, not otherwise specified. In: Swerdlow SH, Campo E, Harris NL et al. eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th ed. Lyon, France: IARC Press; 2008.

Idiopathic Hypereosinophilic Syndrome (I-HES)

I-HES is a diagnosis of exclusion. If the patient has persistent eosinophilia (eosinophil count $\geq 1.5 \times 10^9$/L for at least 6 months) and does not meet the criteria for CEL (Table 24-22) or a familial eosinophilia (autosomal dominant disorder), the diagnosis may be I-HES. I-HES usually has organ-associated involvement and no evidence of clonality or T-cell abnormality (T cells secrete eosinophil cytokines). Pathogenesis is unknown. As more molecular genetic mutations have been found and more diagnostic techniques have become available, this diagnosis has been made less frequently. First-line therapy for most patients with I-HES is the use of corticosteroids.[81] Patients with I-HES should be monitored regularly because follow-up evidence may show that the condition is leukemic.

MAST CELL DISEASE (MASTOCYTOSIS)

Mast cell disorders are heterogeneous and are characterized by the abnormal proliferation of mast cells in one or more organ systems. Mast cells share a common progenitor cell with myelomonocytic cells. See the 2008 WHO classification in Table 24-23 ★.

The two major groups of mast cell disorders are cutaneous and systemic. Cutaneous disease is based on the presence of histological skin lesions and typical clinical signs but no systemic involvement. It is typically found in children.

Systemic disease or **systemic mastocytosis (SM)** involves multifocal histological lesions in the bone marrow and other organs. There may be anemia and an increase or decrease in leukocytes and platelets,and eosinophilia can be marked.

Genetic studies reveal that SM is a clonal disorder characterized by a somatic mutation of the *c-Kit* proto-oncogene. Stem cell factor receptor is encoded for by *c-Kit* (tyrosine kinase receptor) and is a major regulator of mast cell development, and hematopoietic stem cells. When mutated, the receptor is constitutively activated, giving the cell a proliferative advantage.[84]

One subgroup of systemic mast cell disease is mast cell leukemia (MCL), an aggressive disease with a short survival. The criteria for its diagnosis are the fulfillment of the criteria for systemic mastocytosis, circulating mast cells in the peripheral blood (≥10%), mast cells comprising >20% of the nucleated cells in bone marrow, and multiorgan failure (Table 24-24 ★). The aleukemic mast cell leukemia variant has <10% mast cells in the peripheral blood. The bone marrow mast cells can be immature and blast-like. Mast cells with bilobed or polylobed nuclei can be found. Signs of myeloproliferation and dysplasia can be present, but the criteria for another hematologic disorder are not. The mast cells are tryptase positive and express c-Kit. Serum tryptase levels are elevated. Pancytopenia characterizes later stages of the disease when bone marrow failure occurs.

Most patients are adults with symptoms related to proteins or mediators released from mast cells, which include hypotension, flushing, and diarrhea. Weight loss, bone pain, and organomegaly occur later in the disease. No standard therapy or long-term cure exists. Antimediator drugs such as aspirin and antihistamines are used to relieve symptoms. Experience with chemotherapy and bone marrow transplant is lacking.[85]

★ **TABLE 24-23** WHO Classification of Mast Cell Disorders (Mastocytosis)[84,85]

- Cutaneous mastocytosis
- Indolent systemic mastocytosis
- Systemic mastocytosis associated with other clonal, nonmast cell lineage disease
- Aggressive systemic mastocytosis
- Mast cell leukemia
- Mast cell sarcoma
- Extracutaneous mastocytoma

★ **TABLE 24-24** Criteria for Diagnosis of Systemic Mastocytosis (SM) and Mast Cell Leukemia (MCL)

SM requires the major criteria and one minor criteria or three minor criteria. MCL requires criteria for SM plus those listed for MCL in this table.	
Major criteria	• Bone marrow or other organs that show multifocal, dense infiltrates of tryptase positive mast cells (>15 cells in aggregates)
Minor criteria	• Biopsy of bone marrow or other organs shows spindle-shaped or atypical morphology in >25% of mast cells or >25% of all mast cells in the bone marrow are immature or atypical
	• Presence of *c-Kit* point mutation (codon 816) in mast cells from bone marrow or other organ
	• Mast cells in peripheral blood, bone marrow, or other tissue coexpress CD2 and/or CD25 in addition to normal mast cell markers
	• In absence of associated clonal myeloid disorder, total serum tryptase is >20 ng/mL
Mast cell leukemia criteria	• Criteria for SM fulfilled
	• Diffuse infiltration by atypical immature mast cells shown in bone marrow biopsy
	• Bone marrow aspirate with ≥20% mast cells
	Peripheral blood with ≥10% mast cells

Summary

The myeloproliferative neoplasms (MPNs) are characterized as a group of clonal stem cell disorders differentiated by neoplastic production of one or more of the hematopoietic lineages in bone marrow and peripheral blood. The WHO classification of MPN includes the subgroups chronic myelogenous leukemia (CML); chronic neutrophilic leukemia (CNL); essential thrombocythemia (ET); polycythemia vera (PV); primary myelofibrosis (PMF); myeloproliferative neoplasm, unclassifiable (MPN, U); myeloid and lymphoid neoplasms associated with eosinophilia and *PDGFRA*, *PDGFRB*, or *FGFR1* mutations; chronic eosinophilic leukemia, not otherwise specified (CEL-NOS); and mastocytosis.

Although all hematopoietic cell lineages can be involved in the unregulated proliferation in MPNs, one lineage is usually involved more than the others. The myeloid cells are primarily affected in CML, CNL, and eosinophil disorders; the erythrocytes are affected in PV; the platelets/megakaryocytes are affected in ET; and mast cells are elevated in mastocytosis. The most characteristic finding of PMF is a nonmalignant proliferation of fibroblasts in the bone marrow. Splenomegaly, bone marrow fibrosis, and megakaryocytic hyperplasia are findings common to all subgroups.

The underlying pathophysiology appears to be chromosomal rearrangements that occur in the regions of proto-oncogenes leading to qualitative or quantitative alterations in gene expression and abnormal control of cell growth. Abnormal karyotypes in hematopoietic cells can be found in any of the subgroups. The best-characterized abnormality is the Philadelphia (Ph) chromosome found in all individuals with CML. The Ph chromosome is the result of a translocation of genetic material between chromosomes 9 and 22 [t(9;22) (q34;q11.2)]. Results of molecular testing of CML patients, whether positive or negative for the Ph chromosome, reveals a *BCR/ABL1* fusion gene encoding an abnormal tyrosine kinase protein, p210. The function of the abnormal tyrosine kinase results in the pathogenesis of CML. *JAK2*(V617F) and *MPL*(W515) are common mutations in other MPNs that lead to constitutive phosphorylation, signal transduction, DNA transcription, and decreased apoptosis.

The survival of patients with MPNs varies according to the subgroup and complications of thrombosis. Patients with PV and ET appear to survive longer than patients with CML or PMF. Any of the subgroups can evolve into acute leukemia with or without specific therapy. Currently, stem cell transplantation is the only cure, but few patients qualify for the procedure. However, molecular-targeted therapy and antiplatelet drugs give favorable outcomes and improve prognosis, with imatinib mesylate leading the way.

Review Questions

Level I

1. The most prominent cell line in CML is the: (Objective 2)

   A. erythroid

   B. myeloid

   C. megakaryocyte

   D. fibroblast

2. The most prominent cell line found in polycythemia vera (PV) is: (Objective 2)

   A. erythroid

   B. myeloid

   C. megakaryocyte

   D. fibroblast

3. The Philadelphia chromosome (Ph) results from a translocation of chromosomes: (Objective 4)
   A. 8 and 14
   B. 9 and 22
   C. 12 and 17
   D. 15 and 17
4. The peak age for CML is: (Objective 5)
   A. <5 years
   B. 15–30 years
   C. 40–59 years
   D. ≥60 years
5. The Philadelphia chromosome (Ph) can be found in patients with: (Objective 4)
   A. CML
   B. ALL
   C. ET
   D. PV
6. Which of the following represent a characteristic peripheral blood finding in patients with PMF? (Objective 7)
   A. elliptocytes
   B. dacrocytes
   C. target cells
   D. schistocytes
7. PV can be distinguished from secondary polycythemia by measuring: (Objective 9)
   A. hematocrit
   B. plasma volume
   C. hemoglobin concentration
   D. erythropoietin
8. A 50-year-old man was admitted to the emergency room for chest pain and a blood count was ordered. The results showed erythrocyte count 6.5×10^{12}/L; hematocrit 0.60 L/L (60%); leukocyte count 15×10^9/L; and platelet count 500×10^9/L. These results indicate: (Objective 3)
   A. the need for further investigation of a possible diagnosis of MPN
   B. normal findings for an adult male
   C. the patient has experienced a thrombotic episode
   D. a malfunction of the cell-counting instrument
9. A patient previously diagnosed with CML now has a platelet count of 540×10^9/L and a leukocyte count of 350×10^9/L with a peripheral blood differential showing 15% segmented neutrophils, 23% bands, 2% metamyelocytes, 35% blasts, 6% lymphocytes, 4% monocytes, 6% eosinophils, and 8% basophils. These results are most consistent with: (Objective 6)
   A. CML
   B. ET
   C. CML in blast crisis
   D. PMF
10. A patient presenting in the ER with a platelet count of $>1000 \times 10^9$/L and a leukocyte count of 25×10^9/L with a normochromic, normocytic anemia should be evaluated for: (Objective 10)
    A. Ph chromosome
    B. essential thrombocythemia
    C. PMF
    D. primary polycythemia

Level II

Use this case study to answer questions 1–5

A 45-year-old Caucasian female was admitted to the hospital from the emergency room. She had experienced pain in the upper quadrant and bloating for the past several weeks. She had multiple bruises on her legs and arms. She also stated that her gums bled easily when she brushed her teeth. She had been unusually tired and lost about 10 lb in the last 2 months. Results of physical examination showed a massive spleen. The following laboratory results were noted on admission.

		WBC Differential
Hb	7.4 g/dL (74 g/L)	
Erythrocyte count	2.9×10^{12}/L	31% segmented neutrophils
Hct	0.22 L/L (22%)	26% bands
RDW	18.0	8% metamyelocytes
Leukocyte count	520×10^9/L	11% myelocytes
Platelet count	960×10^9/L	4% promyelocytes
		2% blasts
		4% lymphocytes
		3% monocytes
		5% eosinophils
		6% basophils
		4 nucleated erythrocytes/100 leukocytes
		Occasional micromegakaryocytes

Anisocytosis and poikilocytosis were moderate.

A bone marrow aspiration was performed. The marrow was 90% cellular with a myeloid-to-erythroid ratio of 10:1. The majority of the cells were neutrophilic precursors. Eosinophils and basophils were increased. Myeloblasts accounted for 10% of the nucleated cells. Megakaryocytes were increased.

1. What findings suggest that this patient has a defect in the pluripotential stem cell rather than a benign proliferation of hematopoietic cells? (Objective 4)
   A. the presence of a leukoerythroblastic blood picture
   B. the involvement of several cell lineages in the proliferative process including neutrophilic cells and platelets
   C. the shift to the left in the neutrophilic cell line
   D. an increase in the RDW
2. Molecular analysis (RT-PCR) revealed the presence of a *BCR/ABL1* fusion product. Based on this information, what myeloproliferative disorder is present? (Objectives 1, 3)
   A. CML
   B. PV
   C. ET
   D. PMF
3. What cytochemical stain is used to help differentiate a leukemoid reaction from CML? (Objectives 1, 4)
   A. myeloperoxidase
   B. new methylene blue
   C. leukocyte alkaline phosphatase
   D. Perl's Prussian blue
4. Which of the following terms most accurately describes this patient's peripheral blood picture? (Objectives 10, 13)
   A. leukemoid reaction
   B. leukoerythroblastic
   C. leukopenia
   D. myelodysplastic
5. What is the best description of the bone marrow? (Objective 1)
   A. decreased M:E ratio and increased cellularity
   B. increased M:E ratio and decreased cellularity
   C. increased M:E ratio and increased cellularity
   D. decreased M:E ratio and decreased cellularity
6. Extensive bone marrow fibrosis, leukoerythroblastic peripheral blood, and the presence of anisocytosis with dacryocytes are most characteristic of which MPN? (Objective 6)
   A. CML
   B. PV
   C. ET
   D. PMF
7. A 68-year-old man was seen in the clinic for lethargy, dyspnea, and light-headedness. Results of his blood counts were erythrocyte count 5.0×10^{12}/L; hematocrit 0.55 L/L (55%); leukocyte count 60×10^{9}/L; platelet count 70×10^{9}/L. His differential showed a shift to the left in myeloid elements with 40% eosinophils. The bone marrow revealed 10% blasts. Ph chromosome was negative. He most likely has: (Objective 10)
   A. PV
   B. CML in blast crisis
   C. essential thrombocythemia
   D. chronic eosinophilic leukemia
8. A molecular test should be performed on the patient in question 7 for which mutation? (Objective 3)
   A. *JAK2*(V617F)
   B. *BCR/ABL1*
   C. *FIP1L1/PDGFRA*
   D. *MPL*(W515)
9. Which of the following does *not* cause secondary polycythemia? (Objective 7)
   A. chronic obstructive pulmonary disease
   B. smoking
   C. emphysema
   D. dehydration
10. Which of the following gene mutations are uniquely associated with clonal hypereosinophilia? (Objective 11)
    A. *JAK2*(V617F)
    B. *F1P1L1/PDGFRA*
    C. *MPL*(W515)
    D. *BCR/ABL1*

Companion Resources

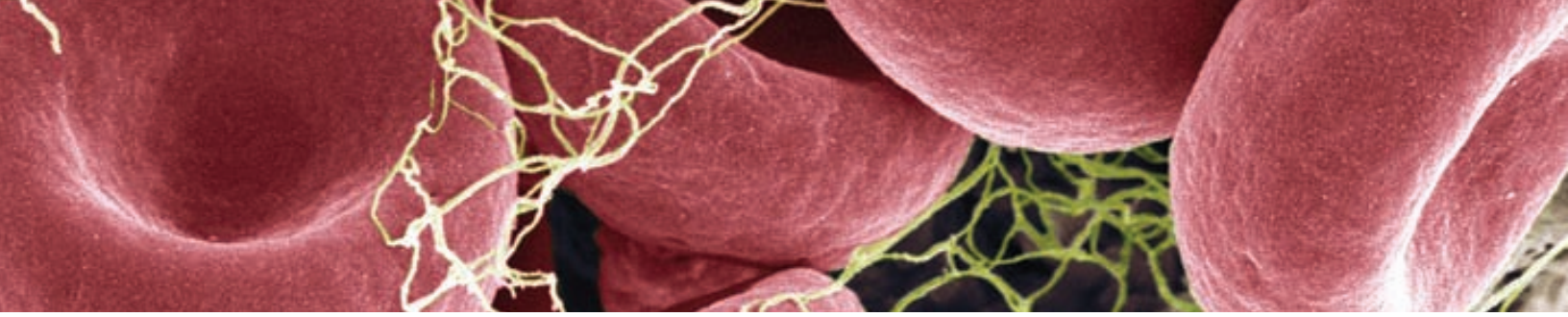
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Dameshek W. Some speculations on the myeloproliferative syndromes. *Blood*. 1951;6:372–75.
2. Gaman M, Vladareanu AM, Radesi S. The implications of revised WHO classification (2008) of chronic myeloid neoplasms. *Rom J Intern Med*. 2011;49(1):25–30.
3. Them NC, Kralovics R. Genetic basis of MPN: beyond JAK2-V617F. *Curr Hematol Malig Rep*. 2013;8:299-306.
4. Cervantes F, Salgado C, Rozman C. Assessment of iron stores in hospitalized patients. *Am J Clin Pathol*. 1991;1:105–6.
5. Bleeker JS, Hogan WJ. Thrombocytosis: diagnostic evaluation, thrombotic risk stratification, and risk-based management strategies. *Thrombosis*. 2011;2011:1–16.
6. Kaushansky K. Hematopoietic growth factors, signaling and the chronic myeloproliferative disorders. *Cytokine Growth Factor Rev*. 2006;17:423–30.
7. Dickstein JI, Vardiman JW. Issues in the pathology and diagnosis of the chronic myeloproliferative disorders and the myelodysplastic syndromes. *Am J Clin Pathol*. 1993;99:513–25.
8. Brazma D, Grace C, Howard J et al. Genomic profile of chronic myelogenous leukemia: imbalances associated with disease progression. *Genes Chromosomes Cancer*. 2007;46:1039–50.
9. Liesveld JL, Lichtman MA. Chronic myelogenous leukemia and related disorders. In: Prchal JT, Kaushansky K, Lichtman MA et al, eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010: 1331–80.
10. Foroni L, Gerrar G, Nna E, et al. Technical aspects and clinical applications of measuring BCR-ABL1 transcripts number in chronic myeloid leukemia. Am J Hematol. 2009; 84:517–22.
11. Vakil, E, Tefferi A. BCR-ABL1—negative myeloproliferative neoplasms: a review of molecular biology, diagnosis, and treatment. *Clin Lymphoma Myeloma Leuk*. 2011;(Suppl 1)1:37–45.
12. Yilmaz M, Kantarjian H, Jabbour E et al. Similar outcome of patients with chronic myeloid leukemia treated with imatinib in or out of clinical trials. *Clin Lymphoma Myeloma Leuk*. 2013;13:693–9.
13. Nadal E, Olavarria E. Imatinib mesylate (Gleevec/Glivec) a molecular-targeted therapy for chronic myeloid leukaemia and other malignancies. *Int J Clin Pract*. 2004;58:511–16.
14. Milojkovic D, Apperley J. Mechanisms of resistance to imatinib and second-generation tyrosine inhibitors in chronic myeloid leukemia. *Clin Cancer Res*. 2009;15:7519–27.
15. Zhou T, Parillon L, Li F et al. T315I ABL mutant-threonine mutated to isoleucine at gatekeeper position of kinase domain. *Chem Biol Drug Des*. 2007;70:171–81.
16. Guihot F, Roy L, Tomowiak C. Current treatment strategies in chronic myeloid leukemia. *Curr Opin Hematol*. 2012;19:102–9.
17. Ma W, Tseng R, Gorre M et al. Plasma RNA as an alternative to cells for monitoring molecular response in patients with chronic myeloid leukemia. *Haematologica*. 2007;92:170–75.
18. Hughes T, Deininger M, Hochhaus A et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood*. 2006;108:28–37.
19. Bain BJ, Brunning RD, Vardiman JW et al. Chronic neutrophilic leukemia. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2008: 38–39.
20. Beer PA, Green AR. Essential thrombocythemia. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010; 1237–48.
21. Arellano-Rodrigo E, Alvarez-Larran A, Reverter JC et al. Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with JAK2 mutational status. *Haematologica*. 2006;91:169–75.
22. Darbousset R, Thomas GM, Mezouar S et al. Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. *Blood*. 2012;120:2133–43.
23. Tefferi A, Elliott M. Thrombosis in myeloproliferative disorders: prevalence, prognostic factors, and the role of leukocytes and JAK2V617F. *Semin Thromb Hemost*. 2007;33:313–20.
24. Campbell PJ, Scott LM, Buck G et al. Definition of subtypes of essential thrombocythaemia and relation to polycythemia vera based on JAK2 V617F mutation status: a prospective study. *Lancet*. 2005;366:1945–53.
25. Vainchenker W, Delhommeau F, Constantinescu SN et al. New mutations and pathogenesis of myeloproliferative neoplasms. *Blood*. 2011;118:1723–35.
26. Kittur J, Knudson RA, Lasho TL et al. Clinical correlations of JAK2V617F allele burden in essential thrombocythemia. *Cancer*. 2007;109:2279–84.
27. Dahabreh IJ, Zoi K, Giannouli S et al. Is JAK2 V617F mutation more than a diagnostic index? a meta-analysis of clinical outcomes in essential thrombocythemia. *Leuk Res*. 2009;33:67–73.
28. Vannucchi AM, Antonioli E, Guglielmelli P et al. Clinical profile of homozygous JAK2 617V > F mutation in patients with polycythemia vera or essential thrombocythemia. *Blood*. 2007;110:840–46.
29. Vannucchi AM, Antonioli E, Guglielmelli P et al. Prospective identification of high-risk polycythemia vera patients based on JAK2(V617F) allele burden. *Leukemia*. 2007;21:1952–59.
30. Levine RL, Wenig G. Role of JAK-STAT signaling in the pathogenesis of myeloproliferative disorder. *Hematology Am Soc Hematol Educ Program*. 2006;233–39, 510.
31. Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH, and IKZF1. *Leukemia*. 2010;24:1128–38.
32. Michiels JJ, Berneman Z, Schroyens W et al. Platelet-mediated erythromelalgic, cerebral, ocular and coronary microvascular ischemic and thrombotic manifestations in patients with essential thrombocythemia and polycythemia vera: a distinct aspirin-responsive and coumadin-resistant arterial thrombophilia. *Platelets*. 2006;17:528–44.
33. Imbert M, Vardiman JW, Pierre RV et al. Essential thrombocythaemia. In: Jaffe ES, Harris NL, Stein H et al. eds. *World Health Organization classification of tumours: Tumours of the haematopoietic and lymphoid tissues*. Lyon, France: IARC Press; 2001:27.
34. Barosi G, Birgrgard G, Finazzi G et al. Response criteria for essential thrombocythemia and polycythemia vera: result of European LeukemiaNet consensus conference. *Blood*. 2009;113:4829–33.
35. Verstovsek S, Passamonti F, Rambaldi A et al. A phase 2 study of INCB018424, an oral, selective JAK1/JAK2 inhibitor, in patients with advanced polycythemia vera (PV) and essential thrombocythemia (ET) refractory to hydroxyuria. *Blood*. 2009;113:4829–33.
36. Stein BL, Crispino JD, Moliterno AR. Janus kinase inhibitors: an update on the progress and promise of targeted therapy in the myeloproliferative neoplasms. *Curr Opin Oncol*. 2011;23:609–616.
37. Thiele J, Kvasnicka HM, Orazi A et al. Essential thrombocythaemia. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2008:48–53.
38. Teffari A, Skoda R, Vardiman JW, Myeloproliferative neoplasms: contemporary diagnosis using histology and genetics. Nat Rev Clin Oncol. 2009; 6:627–37.

39. Harrison CN, Bareford D, Butt N, et al. Guideline for investigation and management of adults and children presenting with a thrombocytosis. Br J Haematol. 2010;149:352–75.
40. Prchal JT, Prchal JF. Polycyehemia Vera. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010; 1223–36.
41. Tefferi A, Spivak JL. Polycythemia vera: scientific advances and current practice. *Semin Hematol*. 2005;42:206–20.
42. Tefferi A. Polycythemia vera: a comprehensive review and clinical recommendations. *Mayo Clin Proc*. 2003;78:174–94.
43. Cao M, Olsen RJ, Zu Y. Polycythemia vera: new clinicopathologic perspectives. *Arch Pathol Lab Med*. 2006;130:1126–32.
44. Scott LM, Tong Y, Levine RI et al. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med*. 2007;356:459–68.
45. Kralovics R, Teo SS, Li S et al. Acquisition of the V615F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders. *Blood*. 2006;108:1377–80.
46. Nussenzveig RH, Swierczek SI, Jelinek J et al. Polycythemia vera is not initiated by JAK2V617F mutation. *Exp Hematol*. 2007;35:32–38.
47. Theocharadies A, Boissinot M, Girodon F et al. Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. *Blood*. 2007;110:375–79.
48. Kilpivaara O, Mukherjee S, Schram AM et al. A germline JAK2 SNP is associated with predisposition to the development of JAK2(V617F)-positive myeloproliferative neoplasms. *Nat Genet*. 2009;41:455–59.
49. Ito S, D'Alessio AC, Taranova OV et al. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specifications. *Nature*. 2010;466:1129–33.
50. Ko M, Huang Y, Jankowska AM et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature*. 2010;468:839–43.
51. Saint-Martin C, Leroy G, Delhommeau F et al. Analysis of the ten-eleven translocation 2 (TET2) gene in familial myeloproliferative neoplasms. *Blood*. 2009;114:1628–32.
52. Abdel-Wahab O. Genetics of the myeloproliferative neoplasms. *Cur Opin Hematol*. 2011;18:117–23.
53. Lasho TL, Pardanani A, Tefferi A. LNK mutations in JAK2 mutation-negative erythrocytosis. *N Engl J Med*. 2010;363:1189–90.
54. Dupont S, Masse A, James C et al. The JAK2 617V > F mutation triggers erythropoiesis hypersensitivity and terminal erythroid amplification in primary cells from patients with polycythemia vera. *Blood*. 2007;110:1013–21.
55. Tefferi A, Lasho TL, Abdel-Wahab O et al. IDH1 and IDH2 mutation studies in 1473 patients with chronic-, fibrotic-, or blast-phase essential thrombocythemia, polycythemia vera or myelofibrosis. *Leukemia*. 2010;24:1302–9.
56. Passamonti F, Rumi E, Arcani L et al. Prognostic factors for thrombosis, myelofibrosis, and leukemia in essential thrombocythemia: a study of 605 patients. *Haematologica*. 2008;93:1645–51.
57. Pardanani A, Tefferi A. Targeting myeloproliferative neoplasms with JAK inhibitors. *Curr Opin Hematol*. 2011;18:105–10.
58. Barbui T, Finazzi G. When and how to treat essential thrombocythemia. *N Engl J Med*. 2005;353:85–86.
59. Tefferi A. JAK inhibitors for myeloproliferative neoplasms: clarifying facts from myths. *Blood*. 2012; 19:2721–30.
60. Barosi G, Birgegard G, Finazzi G et al. Response criteria for essential thrombocythemia and polycythemia vera: result of a European LeukemiaNet consensus conference. *Blood*. 2009;113:4829–33.
61. Moliterno AR, Hexner E, Roboz GJ et al. An open-label study of CEP-701 in patients with JAK2V617F-positive PV and ET: update of 39 enrolled patients. *Blood*. 2009;114:abstract 753.
62. Thiele J, Kvasnicka HM, Orazi A et al. Polycythaemia vera. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2008:41–43.
63. Vardiman JW, Thiele J, Arber DA et al. The 2008 revision of the World Health Organization (WHO) classification of the myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–51.
64. Silver RT, Chow W, Orazi A, Evaluation of WHO criteria for diagnosis of polycythemia vera: aprospective analysis. Blood. 2013;122:1881–6.
65. Cassinat B, Laguillier C, Gardin C et al. Classification of myeloproliferative disorders in the JAK2 era: is there a role for red cell mass? *Leukemia*. 2008;22:452–53.
66. Mascarenhas J, Najfeld V, Kremyanskaya M et al. Primary myelofibrosis. In: Hoffman R, Furie B, Benz EJ Jr et al., eds. *Hematology: Basic Principles and Practice*, 5th ed. Philadelphia: Elsevier Churchill Livingstone; 2013:1053–76.
67. Lichtman MA, Tefferi A. Primary myelofibrosis. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010; 1381–1402.
68. Grand FH, Hidalgo-Curtis CE, Ernst T et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. *Blood*. 2009;113:6182–92.
69. Arana-Yi C, Quintas-Cardama A, Giles F et al. Advances in the therapy of chronic idiopathic myelofibrosis. *Oncologist*. 2006;11:929–43.
70. Vannucchi AM, Antonioli E, Guglielmelli P et. al. Clinical correlations of JAK2(V617F) presence or allele burden in myeloproliferative neoplasms: a critical reappraisal. *Leukemia*. 2008;22:1299–307.
71. Tefferi A, Lasho TL, Huang J et. al. Low JAK2V617F allele burden in primary myelofibrosis, compared with either a higher allele burden or unmutated status, is associated with inferior overall and leukemic-free survival. *Leukemia*. 2008;22:756–61.
72. Scott LM, Scott MA, Campbell PJ et al. Progenitors homozygous for the V617F mutation occur in most patients with polycythemia vera but not essential thrombocythemia. *Blood*. 2006;108:2435–37.
73. Guglielmelli P, Barosi G, Specchia G et al. Identification of patients with poorer survival in primary myelofibrosis based on the burden of JAK2V617F mutated allele. *Blood*. 2009;114:1477–83.
74. Mesa RA, Schwager S, Radia D et al. The Myelofibrosis Symptom Assessment Form (MFSAF): an evidence-based brief inventory to measure quality of life and symptomatic response to treatment in myelofibrosis. *Leuk Res*. 2009;33:1199–203.
75. Mesa RA, Niblack J, Wadleigh M et al. The burden of fatigue and quality of life in myeloproliferative disorders (MPDs): an international internet based survey of 1179 MPD patients. *Cancer*. 2007;109:68–76.
76. Pullarkat V, Bass RD, Gong JZ et al. Primary autoimmune myelofibrosis: definition of a distinct clinicopathologic syndrome. *Am J Hematol*. 2003;72:8–12.
77. Gangat N, Caramazza D, Vaidya R et al. DIPSS-Plus: a refined dynamic international prognostic indicator system (DIPSS) for primary myelofibrosis that incorporates prognostic information from karyotype, platelet count and transfusion status. *J Clin Oncol*. 2011;29(4):392–97.
78. Mesa RA, Pardanani AD, Hussein K et al. Phase 1/-2 study of pomalidomide in myelofibrosis. *Am J Hematol*. 2010;85(2):129–30.
79. Verstovsek S, Kantarjian H, Mesa RA et al. Safety and efficacy of INC018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *N Engl J Med*. 2010;363:1117–127.
80. Havelange V, Demoulin JB. Review of current classification, molecular alterations, and tyrosine kinase inhibitor therapies in myeloproliferative disorders with hypereosinophilia. *J Blood Med*. 2013;4:111–21.
81. Klion AD. Recent advances in the diagnosis and treatment of hypereosinophilic syndromes. *Hematology Am Soc Hematol Educ Program*. 2005;209–14.
82. Montes M, Sawhney C, Barros N. Strongyloides stercoralis: there but not seen. *Curr Opin Infect Dis*. 2010;23(5):500–4.
83. Bain BJ, Gilliland DG, Vardiman JW et al. Chronic eosinophilic leukemia, not otherwise specified. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th ed. Lyon: IARC Press. 2008:68–73.
84. Valent P, Horny HP, Escribano L et al. Diagnostic criteria and classification of mastocytosis: a consensus proposal. *Leuk Res*. 2001;25(7):603–25.
85. Horny HP, Metcalfe DD, Bennett JM et al. Mastocytosis. In: Swerdlow SH, Campo E, Harris N et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: IARC Press; 2008:48–53.



25 Myelodysplastic Syndromes

LOUANN W. LAWRENCE, DrPH
SARA A. TAYLOR, PhD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *myelodysplastic syndrome* (*MDS*) and list general characteristics of these diseases.
2. List the six subgroups of MDS recognized by the 2008 World Health Organization (WHO) Classification System and identify key morphological and clinical criteria that distinguish each group.
3. Describe laboratory findings and recognize changes in morphology that are characteristic of this group of disorders.
4. Define the WHO category of myelodysplastic/myeloproliferative neoplasms (MDS/MPNs), and list its general characteristics.
5. List the four subgroups of MDS/MPNs and identify key morphological and clinical criteria as well as laboratory findings that distinguish each group.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Describe the pathophysiology of MDS.
2. Distinguish among agranular blasts, granular blasts, and promyelocytes.
3. Summarize the treatment and prognosis of MDS.
4. Explain the relationship between MDS and acute leukemia.
5. Assess the results of cytogenetic and molecular tests and correlate them with a diagnosis of MDS.
6. List and briefly describe the MDS variants not listed in the six WHO subgroups.
7. Differentiate MDS from MPN, acute leukemia, and other hematologic abnormalities using peripheral blood, bone marrow, and cytogenetic characteristics.
8. Differentiate MDS/MPNs from MDS, MPN, acute leukemia, and other hematologic abnormalities using peripheral blood, bone marrow, and cytogenetic characteristics.
9. Select laboratory tests that are helpful in diagnosing and differentiating MDS and MDS/MPNs.
10. Evaluate a patient's laboratory and clinical findings, and propose a diagnostic MDS or MDS/MPNs subgroup based on those findings.

Chapter Outline

Key Terms

Atypical chronic myeloid leukemia (aCML, *BCR/ABL1*$^-$)
Chronic myelomonocytic leukemia (CMML)
Dysplasia
Dyspoiesis
Endomitosis
Juvenile myelomonocytic leukemia (JMML)
Micromegakaryocyte
Myelodysplastic syndromes (MDS)
Myelodysplastic/myeloproliferative neoplasms (MDS/MPNs)
Refractory anemia with excess blasts (RAEB)
Refractory anemia with ring sideroblasts (RARS)
Refractory cytopenia with multilineage dysplasia (RCMD)
Refractory cytopenia with unilineage dysplasia (RCUD)
Ring sideroblasts

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review the following concepts before starting this unit of study:

Level I

- Create a schematic depicting derivation of different types of blood cells from the pluripotent hematopoietic stem cell. (Chapter 2)
- Describe and recognize the cell morphology described by these terms: ring sideroblasts (Chapter 12), Pelger-Huët anomaly (Chapter 21), and megaloblastoid erythropoiesis. (Chapter 15)
- Explain what *ineffective hematopoiesis* means. (Chapters 11, 24)
- Summarize the history and basis of the classification system to classify malignant leukocyte disorders. (Chapter 23)
- Describe normal leukocyte development, differentiation, and concentrations in the peripheral blood. (Chapter 8)

Level II

- Describe the use of cytochemical and immunological features that distinguish the different types of blasts. (Chapters 23, 40)
- Explain how oncogenes and hematopoietic growth factors affect cellular maturation and proliferation. (Chapters 3, 23)
- Explain the role of epigenetics in cellular development. (Chapter 2)
- Explain the use of cytogenetics and flow cytometry in the diagnosis of malignant disorders. (Chapters 40, 41)

CASE STUDY

We will refer to this case study throughout the chapter.

Hancock, a 65-year-old white male, was seen in triage with complaints of fatigue, malaise, anorexia, and hemoptysis of recent onset. A complete blood count (CBC) was ordered and revealed anemia and a shift to the left in granulocytes. Hematopoietic cells showed dysplastic features.

Consider diagnostic probabilities and reflex testing that could provide differential diagnostic information.

OVERVIEW

This chapter describes the neoplastic hematopoietic disorders known as the *myelodysplastic syndromes (MDS)*. It begins with a discussion of the classification of the disorders into morphologic subgroups according to the 2008 classification of the World Health Organization. This is followed by a description of the pathogenesis, incidence, and general clinical and laboratory features, including peripheral blood and bone marrow findings. Each subgroup is then described with specific features that allow it to be classified. Variants are also described. Genetic findings common to MDS are discussed. The chapter concludes with prognosis and therapy.

INTRODUCTION

The **myelodysplastic syndromes (MDS)** are considered primary, neoplastic, pluripotential stem cell disorders. They are characterized by one or more peripheral blood cytopenias with prominent maturation abnormalities (**dyspoiesis** or **dysplasia**) in the bone marrow. The peripheral blood cytopenias result from ineffective hematopoiesis and increased apoptosis as evidenced by an accompanying bone marrow hyperplasia. These relatively common entities evolve progressively, leading to the aggravation of the cytopenias and, in some cases, transformation into a condition indistinguishable from acute leukemia. MDS occurs most commonly in the elderly, although it is diagnosed occasionally in children.

Before the 1980s, much confusion and disagreement existed in the literature concerning the criteria for defining, subgrouping, and naming MDS. Because of the predisposition of MDS to terminate in leukemia, the term *preleukemia* commonly was used to describe these disorders. However, the evolution of MDS to acute leukemia is not obligatory, and, in fact, many patients die of intercurrent disease or complications of the cytopenia before evolving to leukemia.[1] Whether these patients would have developed leukemia had they survived the cytopenic complications is, of course, unknown. In addition, the diagnosis of preleukemia can be made only in retrospect (i.e., after the patient develops leukemia). Thus, the term *myelodysplasia* is more appropriate until the patient actually develops overt leukemia. In the past, MDS also has

been described under the terms *refractory anemia with excess blasts*, *chronic erythremic myelosis*, *hematopoietic dysplasia*, *refractory anemia with or without sideroblasts*, *subacute or chronic myelomonocytic leukemia*, and *smoldering leukemia*. Most hematologists currently consider the terms *dysmyelopoietic syndrome* and *myelodysplastic syndrome* to be more acceptable than *preleukemia* or other synonyms in describing these hematologic disorders. In this book, the term *myelodysplastic syndrome* is used.

PATHOGENESIS

Myeolyelodysplastic syndromes arise from proliferation of stem cells that have acquired a defect that prevents them from differentiating, maturing, and proliferating normally. The stem cell abnormality most often involves the myeloid progenitor cell; the lymphocytic cell lines are rarely affected. Many of the afflicted stem cells do differentiate into mature cells but exhibit flawed and ineffective progression so that their numbers decrease in the peripheral blood, a phenomenon known as *ineffective hematopoiesis*. In addition to decreased numbers, the myelodysplastic cells develop abnormal morphology and exhibit compromised function. Gradually, the stem cells lose their ability to differentiate, and in some cases they transform into cells that are consistent with acute leukemia.

Myelodysplastic syndromes are described as either primary or secondary in nature. Approximately 60–70% of MDS patients exhibit primary MDS. In these cases, the abnormal stem cell arises de novo—the patient has no known history of exposure to chemotherapy or radiation therapy. However, myelodysplastic syndromes are acquired neoplasms, and environmental insults such as exposure to genotoxic chemicals (benzene), radiation, and viral infection are all etiologic agents that putatively contribute to MDS development.

The dysplastic stem cell of secondary or therapy-related MDS develops after chemotherapy with alkylating agents or topoisomerase II inhibitors either in conjunction with radiation treatment or alone.[2] Most cases of therapy-related MDS occur in patients who have been treated for lymphoma or a solid tumor.

Regardless of whether the MDS is primary or secondary in nature, a multistep pathogenesis is believed to be necessary for MDS to develop. MDS appears to develop as a result of complex interactions between compromised hematopoietic stem cells and the microenvironment of the bone marrow.[3,4] Although nearly 50% of MDS patients present with normal chromosomes, genetic instability of the myeloid stem cell almost certainly contributes to the development of MDS. That MDS is primarily a disease present in elderly patients lends credence to the implication of genomic instability as a contributing factor toward disease progression.

☑ CHECKPOINT 25-1

How does the pathogenesis of primary MDS differ from secondary MDS?

Cytogenetics, Epigenetics, and Single Gene Mutations

The abnormal MDS clone is characterized by altered function of genes that result from chromosomal abnormalities, gene silencing (epigenetic changes), or single gene mutations. "Unbalanced" genetic abnormalities (which result in a larger or smaller amount of DNA in the cell, such as trisomies or whole or partial chromosomal deletions) are characteristically seen in the MDS and are thought to be responsible for the ineffective hematopoiesis and cytopenias.[2] These chromosomal abnormalities are in contrast to the balanced abnormalities that maintain the normal quantity of DNA (e.g., reciprocal translocations) seen primarily in myeloproliferative neoplasms (MPNs) and acute myeloid leukemia (AML) (Chapters 24 and 26, respectively). Furthermore, many chromosomal alterations involve gene deletions, suggesting that tumor suppressor genes or DNA repair genes are altered (Chapter 23).

Abnormal Karyotypes

Nearly every chromosome has been shown to exhibit genomic aberration in MDS. Moreover, abnormal cytogenetic patterns in MDS are heterogeneous; chromosome abnormalities can present either as the sole aberration in MDS or can occur as part of a complex karyotype. The most commonly encountered abnormal karyotypes in MDS are the −5/del(5q),−7/del(7q), +8, del(20q), and −Y, suggesting that genes located in these regions contribute to the pathogenesis of this syndrome.[5–7] Table 25-1 ★ describes the most frequently mutated genes in MDS.

−5 and del(5q)

−5 and del(5q) are among the most frequently noted genetic abnormalities in MDS, found in 10–20% of myelodysplastic syndromes.[2,6] The WHO classification of myeloid neoplasms includes an MDS subtype named for this cytogenetic abnormality when it exists as the sole aberration: MDS associated with isolated del(5q).

Three commonly deleted regions (CDRs) are found on the long arm of chromosome 5, located among bands 5q31–33.[2,8] The loss of many of the genes in these CDRs seems to have a role in the pathogenesis of hematopoietic malignancies; their mechanisms of action include the control of cell signaling, transcription, cell cycle, antioxidant levels, apoptosis, cell differentiation/proliferation, and tumor suppression. Specific genes located on chromosome 5 that commonly are lost with a complete or partial deletion of chromosome 5 include *EGR1* (early growth response gene 1), *NPM1*, (nucleophosmin 1), and *RPS14* (encodes a ribosomal subunit protein). Whole or partial losses of chromosome 5 are mostly somatically acquired; often they are associated with earlier treatment with radiotherapy, alkylating agents, or DNA topoisomerase II antagonists.[6]

−7 and del(7q)

Karyotypic abnormalities on chromosome 7 appear with up to 20% incidence in MDS patients.[2,6] The −7 karyotype presents with severe refractory cytopenias and increased incidence of severe infections.[6] Three distinct CDRs have been identified on chromosome 7, one at 7q22, and the other two at 7q31–32 and 7q36.[9,10] It is very likely that the long arm of chromosome 7 is well populated with tumor suppressor genes, although such specific genes have not yet been identified.

+8

Trisomy 8 occurs with about 10% frequency in MDS. In contrast to whole or partial losses of chromosomes 5 and 7, trisomy 8 in AML and MDS is not associated with prior treatment with radiation, alkylating

★ **TABLE 25-1** Significant Mutated Genes in MDS

Gene	Location	Frequency	Significance
ASXL1	20q11.1	~11%	Putative function in myeloproliferation, more prevalent in MDS/MPN overlap syndromes
EGR1	5q31	5–10%	Putative tumor suppressor gene, haploinsufficiency suggested as an initiating event in MDS
EVI1	3q26.2	~5%	Tumor suppressor signaling and prevention of apoptosis
FLT3	13q12	5–10%	Encodes for a tyrosine kinase, regulates proliferation, differentiation, apoptosis in hematopoietic cells, mutations associated with progression to AML
JAK2	9p	5% 60% in RARS-t	Activating *JAK2* mutations cause cellular hypersensitivity to cytokines
NPM1	5(q35)	5%	Putative proto-oncogene that inactivates p53
N-RAS	1p13.2	Up to 15% in MDS	Activating mutations stimulate MAPK signaling
K-RAS	12p12.1	Up to 40% in advanced MDS	Associated with a worse prognosis and higher likelihood of progression to AML
RUNX1	21q22.12	20%	Important for hematopoietic differentiation
p53	17p13.1	20%; 25% in secondary MDS	Encodes a checkpoint protein (p53), rapid progression to AML and poor prognosis

ASXL1 = additional sex combs like 1; *EGR1* = early-growth response gene 1; *EVI1* = ecotropic viral integration site 1; *FLT3* = fms-related tyrosine kinase 3; *JAK2* = janus kinase 2; *NPM1* = nuclephosmin 1; *N-RAS* = neuroblastoma rat sarcoma; *K- RAS* = Kirsten rat sarcoma; *RUNX1* = runt-related transcription factor 1

agents, or DNA topoisomerse II antagonists.[11,12] Little is known about the pathogenetic significance of trisomy 8 in myeloid malignancies although it has been suggested that its effect is largely influenced by the overexpression of the proto-oncogene *MYC* at 8q24.[13]

del(20q)

A deletion of the long arm of chromosome 20 is seen in about 5% of primary MDS and in about 7% of secondary MDS. The *L3MBTL1* gene encodes a transcriptional repressor and is located in this deleted region. The functions of the L3MBTL1 protein are not fully elucidated, but it has been shown to be involved in chromosome compaction (see the section "Epigenetic Changes") and to inhibit the expression of the *RUNX1* gene (Table 25-1). Loss of the *L3MBTL1* gene in patients carrying del(20q) is likely to be involved in the neoplastic state of MDS.[14]

−Y

MDS patients can present with a loss of the Y chromosome, but the frequency is variable, and the significance of the missing chromosome remains uncertain. −Y is found in many malignant diseases but also in healthy, aging men.

Complex Karyotype

Approximately 20% of patients exhibiting primary MDS and nearly 90% of MDS patients diagnosed with treatment-related MDS exhibit a complex karyotype. Complex karyotypes generally involve the presence of more than three chromosomal abnormalities. Whole or partial deletions of chromosome 5, chromosome 7, or both are frequently components of a complex karyotype.[9]

Epigenetic Changes

Epigenetic changes such as DNA methylation and histone modifications are frequently observed in MDS.[15,16] DNA methylation consists of the addition of a methyl group, most often to cytosine in regions containing numerous cytosines and guanines (termed *CpG islands*; *p* represents the phosphodiester bond of DNA). These regions are commonly found within gene promoter regions. CpG hypermethylation silences the transcript.

Multiple genes, including those for cell cycle regulators, tumor suppressors, DNA repair, and apoptosis, have been identified as targets for methylation.[8,15] Silencing any of these critical genes could contribute to the development of MDS. Certainly, DNA methylation is a significant factor in the progression of MDS into AML.[17,18]

Most studies of the significance of epigenetic modification in MDS have focused on DNA methylation, but histone modification also is being investigated as a putative factor. Histone acetylation relaxes DNA and allows the proteins that control gene expression access to the gene site, thus allowing genes to be expressed. Histone methylation can be either repressive or activating, depending on the gene involved. The influence of epigenetic changes in the development of MDS will certainly be more fully elucidated in the future. Treatment with drugs that modify epigenetic transformation are examined in the section on MDS treatment.

Oncogenes and Tumor Suppressor Genes

Control of cellular differentiation, maturation, and proliferation occurs through properly functioning proto-oncogenes and tumor suppressor genes (Chapter 23) and when these genes are mutated, neoplasia can result.[3] Although mutations in the proto-oncogenes *JAK2* (Chapter 24), *RAS*, and *RUNX1* and the tumor suppressor gene *p53* are most commonly observed, the overall incidence of single gene mutations is low[4] (Table 25-1).

N- and *K-RAS* mutations have been observed in 10–15% of patients with MDS. The most common mutation is a single base change in codon 12 of the *N-RAS* (neuroblastoma-ras) family. RAS proteins normally function in growth factor–mediated signaling, and mutation is associated with a higher risk of AML transformation and a worse prognosis. *RUNX1* is a regulatory transcription factor for hematopoietic differentiation, and point mutations have been observed in 20% of MDS patients.

Mutations in the tumor suppressor gene *p53* (Chapter 23) appear in approximately 20% of MDS patients and are most commonly associated with secondary MDS. Normal p53 protein functions to maintain genetic stability in cells and *p53* mutations in MDS patients are linked to a poor prognosis.

Proliferation Abnormalities

In vitro studies suggest that excessive premature intramedullary cell death of hematologic precursors via apoptosis contributes to ineffective hematopoiesis and peripheral blood cytopenias seen in early MDS.

☑ CHECKPOINT 25-2

How do epigenetics contribute to the pathogenesis of MDS?

The various gene alterations of the MDS clone result in an intrinsic increase in susceptibility of the clone to apoptosis.[19] Increased levels of tumor necrosis factor (TNF)-α in MDS patients has been linked to increased apoptosis of the MDS clone and normal hematopoietic cells.[19] Progression to leukemia is associated with a reduction in apoptosis, thereby allowing the expansion of the neoplastic clone.

INCIDENCE

MDS occurs primarily in individuals older than 50 years of age and has a slight male predominance by a factor of approximately 1.5. The risk increases sharply with age.[20] The incidence rate in the United States is 4.4 per 100,000.[21] The incidence of MDS increases with age, and the median age at diagnosis is 72 years.[22] The frequency of MDS diagnoses seems to be rising. This rise could be the result of an increase in the awareness of MDS on the part of physicians and clinical laboratory professionals, in the application of diagnostic procedures in elderly individuals, or in the number of elderly in the population.

Myelodysplasia is rare in children. Problems in diagnosis can contribute to an underestimation of incidence. The approximate incidence has been reported as 1.0–1.8 cases/million/year.[19,21] The median age of children at presentation of MDS is about 6 years, and the male-to-female ratio is about 1.6:1.

CLINICAL FINDINGS

The most frequent presenting symptoms, fatigue and weakness, are related to an anemia that is nonresponsive to treatment. Less commonly, hemorrhagic symptoms and infection precede diagnosis. These symptoms are related to thrombocytopenia and neutropenia. Many individuals can be asymptomatic, and their disease is discovered on routine laboratory screening.[23] Infection is a common complication in patients diagnosed with MDS, particularly for those patients diagnosed with an aggressive MDS subgroup or who present with neutropenia ($<1 \times 10^9$/L). Infection and bleeding are the most common causes of death.[22] Splenomegaly and/or hepatomegaly are uncommon.

LABORATORY FINDINGS

MDS presents with a range of abnormal morphologic features that can be demonstrated on stained peripheral blood and bone marrow smears. Criteria for classification of MDS into subtypes based on morphology and cell counts are presented in a later section of this chapter. Included here are the general hematologic features used to initially define the presence of an MDS (Table 25-2 ★).

Peripheral Blood

The peripheral blood characteristics of MDS are cytopenias and dysplasia. Cytopenias are defined as hemoglobin <10 g/dL, absolute neutrophil count $<1.8 \times 10^9$/L, and platelets $<100 \times 10^9$/L.[3] Anemia is the most consistent finding, occurring in $>80\%$ of the cases. Bicytopenia occurs in 30% of the cases and pancytopenia in 15%.[3] Less commonly, an isolated neutropenia or thrombocytopenia is found. Dysplastic features of one or more cell lines are typical. Because of the critical importance of recognizing dysplasia in the diagnosis of MDS, smears should be made from samples that have been anticoagulated for 2 hours or less.[24] Morphologic changes from prolonged exposure to anticoagulant can easily be confused with dysplastic changes in cells. Functional abnormalities of hematologic cells are also common. Studies show that the higher the degree and number of cytopenias, the worse the prognosis.[22]

Erythrocytes

The degree of anemia is variable, but the hemoglobin is <10 g/dL. The erythrocytes are usually macrocytic (Figure 25-1 ■) and less often normocytic. Oval macrocytes similar to those in megaloblastic anemia

★ **TABLE 25-2** Hematologic Abnormalities in Myelodysplastic Syndromes

Findings	Erythroid Series	Myeloid Series	Thrombocyte Series
Peripheral blood	Anemia	Neutropenia	Thrombocytopenia or thrombocytosis
	Anisocytosis, dimorphism	Hypogranulation, abnormal granulation	Giant forms
	Poikilocytosis	Shift to the left	Hypogranulation
	Macrocytes, oval macrocytes	Nuclear abnormalities	Micromegakaryocytes
	Basophilic stippling	Pseudo–Pelger-Huët	Functional abnormalities
	Nucleated RBCs	Ring nuclei	
	Howell-Jolly bodies	Monocytosis	
	Sideroblasts		
	Reticulocytopenia		
Bone marrow	Megaloblastoid erythropoiesis	Abnormal granules in promyelocytes	Micromegakaryocytes
	Nuclear fragmentation and budding	Increase in granular and agranular blasts	Megakaryocytes with multiple separated nuclei
	Karyorrhexis	Absence of secondary granules	Large mononuclear megakaryocytes
	Multiple nuclei	Nuclear abnormalities	Hypogranulation or large abnormal granules in megakaryocytes
	Defective hemoglobinization	Decreased myeloperoxidase	
	Vacuolization	Auer rods in blasts	
	Ring sideroblasts		

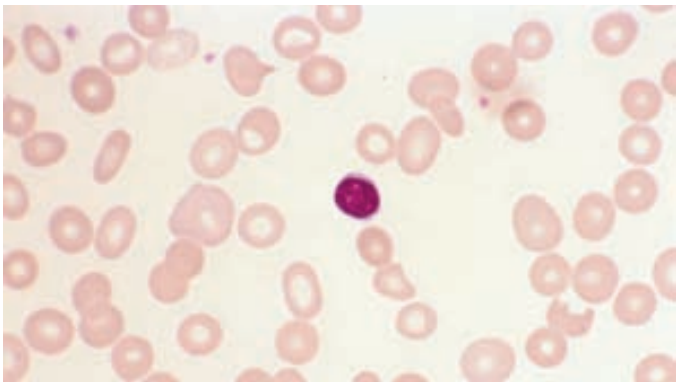


FIGURE 25-1 A peripheral blood film of a patient with MDS. Note macrocytic cells and anisocytosis (peripheral blood, Wright-Giemsa stain, 1000× magnification).

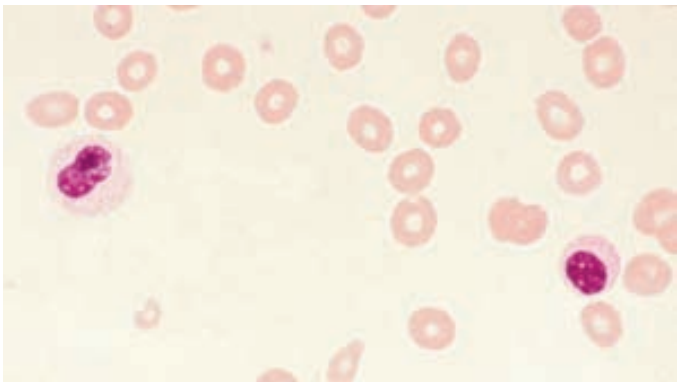


FIGURE 25-2 Peripheral blood film from a patient with MDS showing neutrophils with the pseudo–Pelger-Huët nucleus. One cell's nucleus is peanut shaped and the other is single lobed. The nuclear chromatin is condensed, and the cells contain granules, making identification possible. In many cases, these types of neutrophils are agranular, making differentiation of them from lymphocytes difficult (peripheral blood, Wright-Giemsa stain, 1000× magnification).

are frequently present, but patients have normal vitamin B_{12} and folate levels. A dimorphic anemia with both oval macrocytes or normocytes and microcytic hypochromic cells is also a common initial finding in the refractory anemia with ring sideroblasts (RARS) subgroup. Reticulocytes show an absolute decrease in number but can appear normal if only the uncorrected or relative number (percent) is reported.

In addition to anemia, qualitative abnormalities indicative of dyserythropoiesis are present. These include anisocytosis, poikilocytosis, basophilic stippling, Howell-Jolly bodies, and nucleated erythrocytes. Often hemoglobin F is increased (5–6%) and distributed in a heterogeneous pattern. Acquired hemoglobin H has also been found in MDS.[19] Other erythrocyte changes include altered A, B, and I antigens, enzyme changes, and an acquired erythrocyte membrane change similar but not identical to that found in paroxysmal nocturnal hemoglobinuria (PNH)[19,23] (Chapter 17).

Leukocytes

Neutropenia is the second most common cytopenia observed in MDS and is present in approximately 50% of patients at the time of diagnosis.[19] Neutropenia can be accompanied by a shift to the left with the finding of metamyelocytes and myelocytes on the peripheral blood smear. Blasts and promyelocytes can also be present.

Morphologic abnormalities in granulocytes indicative of dysgranulopoiesis are considered a hallmark finding in MDS. Dysgranulopoiesis is characterized by agranular or hypogranular neutrophils, persistent basophilia of the cytoplasm, abnormal appearing granules, hyposegmentation (pseudo–Pelger-Huët) (Figure 25-2 ■), or hypersegmentation of the nucleus and donut- or ring-shaped nuclei. Care should be taken to distinguish neutrophils with the pseudo–Pelger-Huët anomaly and hypogranulation from lymphocytes and to distinguish neutrophilic-band forms with hypogranulation from monocytes. Neutrophils can also demonstrate enzyme defects, such as decreased myeloperoxidase (MPO) and decreased leukocyte alkaline phosphatase (LAP). In some cases, neutrophils exhibit severe functional impairment, including defective bactericidal, phagocytic, or chemotactic properties. Absolute monocytosis is a common finding even in leukopenic conditions.

Platelets

Qualitative and quantitative platelet abnormalities are often present. The platelet count can be normal, increased, or decreased. Approximately 25–50% of patients have mild to moderate thrombocytopenia when diagnosed.[19] Giant platelets, hypogranular platelets, and platelets with large fused granules can be seen in the peripheral blood (Figures 25-3 ■ and 25-4 ■). Functional platelet abnormalities include abnormal adhesion and aggregation. As a result, platelet function tests can be abnormal.

Micromegakaryocytes

Sometimes circulating **micromegakaryocytes** (small abnormal megakaryocytes), also called *dwarf megakaryocytes*, can be found in MDS and MPNs. Micromegakaryocytes can be difficult to identify and are frequently overlooked unless cytoplasmic tags or blebs are present (Figure 25-5 ■). Micromegakaryocytes are believed to represent abnormal megakaryocytes that have reduced ability to undergo **endomitosis** (megakaryocytes undergo a unique maturation process whereby the DNA content duplicates without cell division, resulting in a polyploid nucleus [endomitosis] [Chapter 31]). Most micromegakaryocytes have a single-lobed nucleus and are the size of a lymphocyte. Morphologically, they can be confused with lymphocytes but can be distinguished by cytoplasmic tags of one or more platelets attached to a nucleus. Some can have pale blue, foamy, or vacuolated cytoplasm that resembles a nongranular platelet. The nuclear structure is variable, but many cells have very densely clumped chromatin and stain dark

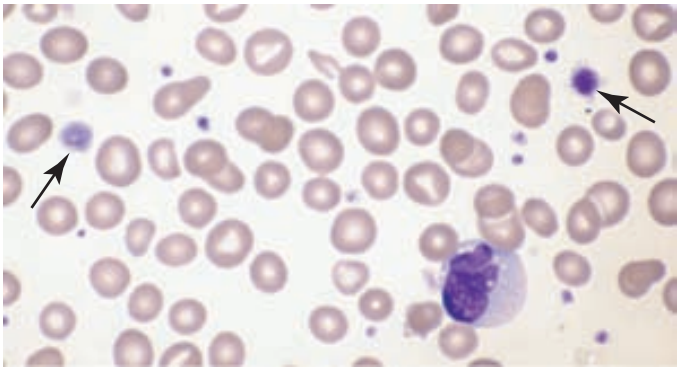


FIGURE 25-3 Arrows point to large platelets in a peripheral blood film from a patient with MDS (Wright-Giemsa stain, 1000× magnification).

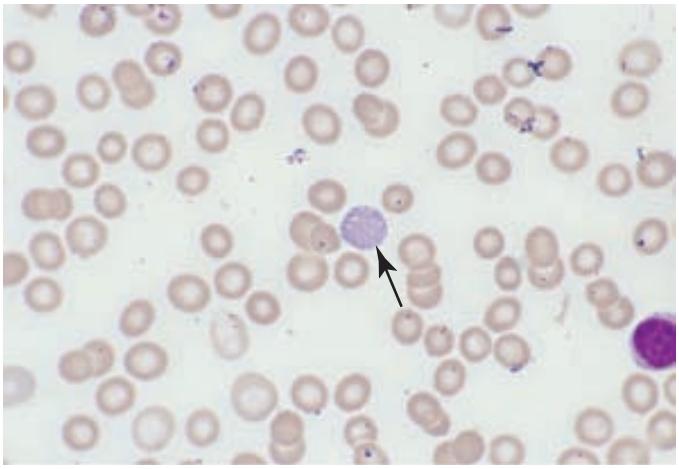


FIGURE 25-4 Arrow points to a large agranular platelet in a peripheral blood film from a patient with MDS (Wright-Giemsa stain, 1000× magnification).

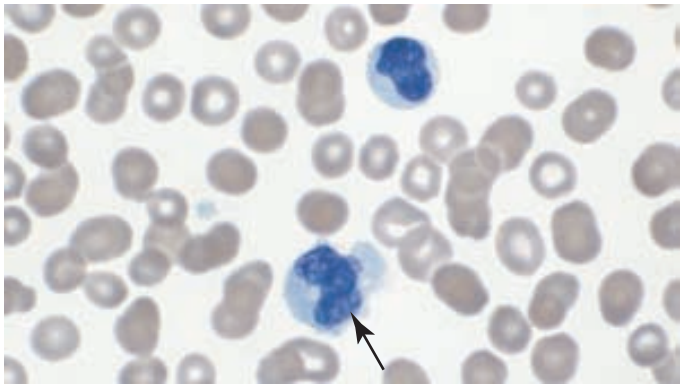


FIGURE 25-5 Arrow points to a micromegakaryocyte in a peripheral blood film from a patient with MDS. Note the dense chromatin structure and the irregular rim of cytoplasm with cytoplasmic tags. An agranular platelet in the field matches the appearance of the micromegakaryocyte cytoplasm (Wright-Giemsa stain, 1000× magnification).

☑ CHECKPOINT 25-3

How does the typical peripheral blood picture in MDS differ from that in aplastic anemia (Chapter 16)?

CASE STUDY *(continued from page 480)*

The results of the CBC on Hancock were:

RBC	1.60 × 10^{12}/L	**WBC Differential**
Hb	5.8 g/dL (58 g/L)	44% segmented neutrophils
Hct	0.17 L/L (17%)	7% band neutrophils
WBC	10.5 × 10^{9}/L	6% lymphocytes
Platelets	39 × 10^{9}/L	28% eosinophils
Reticulocyte count	0.8%	1% metamyelocytes
		1% myelocytes
		9% promyelocytes
		4% blasts

The neutrophilic cells show marked hyposegmentation and hypogranulation. Red blood cell (RBC) morphology includes anisocytosis and poikilocytosis, teardrop cells, ovalocytes, and schistocytes.

1. In what cell lines is cytopenia present?
2. What abnormalities are present in the differential?
3. What evidence of dyspoiesis is seen in the leukocyte morphology?
4. Calculate the mean cell volume (MCV). What peripheral blood findings are helpful to rule out megaloblastic anemia?
5. What features of the differential resemble chronic myeloid leukemia (CML)? What helps to distinguish this case from CML?

blue-black with Wright-Giemsa stain. Others can have a finer, looser chromatin. These cells also are found in the bone marrow.

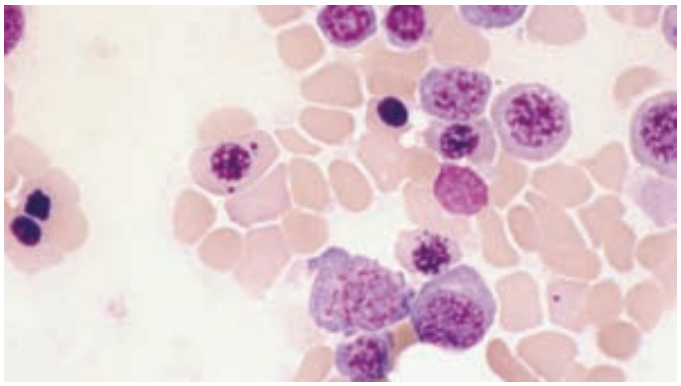
Bone Marrow

Bone marrow examination is necessary to identify the dyshematopoietic element, to determine cellularity, and to establish a diagnosis. In most cases, the bone marrow is hypercellular with erythroid hyperplasia, although normocellular and hypocellular marrows can occur. The hypercellular bone marrow with a peripheral blood picture of cytopenia is the result of premature cell loss in the marrow (increased apoptosis and ineffective hematopoiesis).[19] The cellularity of the marrow should be interpreted in relation to the patient's age because MDS is commonly found in people who are elderly. The number of myeloblasts is <20%. Generally, all cell lines exhibit evidence of dyshematopoiesis. At least 10% of all cells of a given hematopoietic lineage should be dysplastic to allow for the diagnosis of MDS.[3]

Bone marrow biopsy can be helpful in establishing the diagnosis of MDS in difficult cases. Abnormal localization of immature myeloid precursors (ALIP) clustering centrally can be seen in biopsy before an increase in myeloblasts is detected in bone marrow smears.[25,26] ALIP has been shown to indicate increased risk for transformation to leukemia and is associated with poor survival. In patients with <5% blasts, ALIP can indicate evolution to a more aggressive disease. However, these claims have not been validated in large studies.[23] A biopsy also provides a better assessment of cellularity and an indication of the amount of reticulin fibers present. On the other hand, ring sideroblasts, nuclear fragmentation and budding, Auer rods, irregular cytoplasmic basophilia, and abnormal staining of primary granules in promyelocytes are more easily identified in bone marrow aspirate smears. Thus, both aspirate smears and biopsy preparations are necessary for accurate diagnosis.

Dyserythropoiesis

The most common bone marrow finding in MDS is nuclear-cytoplasmic asynchrony similar to that seen in megaloblastic anemia (Chapter 15). However, the chromatin is usually hypercondensed. This nuclear chromatin pattern is often described as megaloblastoid (Figure 25-6 ■). The abnormal erythrocytic maturation is not responsive to vitamin B_{12} or

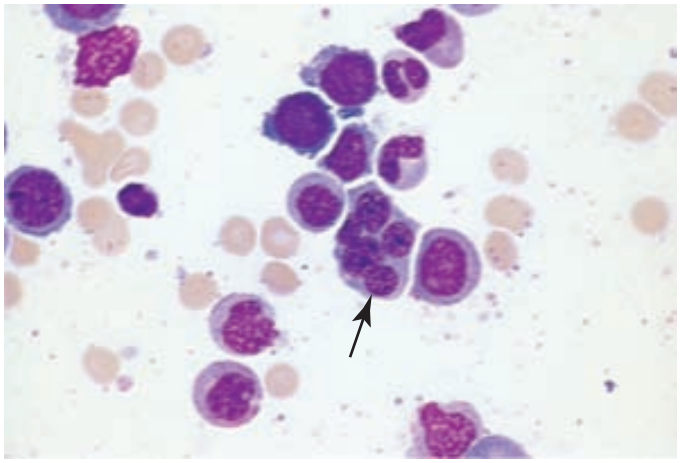


■ **FIGURE 25-6** Megaloblastoid erythroblasts in the bone marrow of a patient with MDS. Note the condensed chromatin and Howell-Jolly bodies (Wright-Giemsa stain, 1000× magnification).

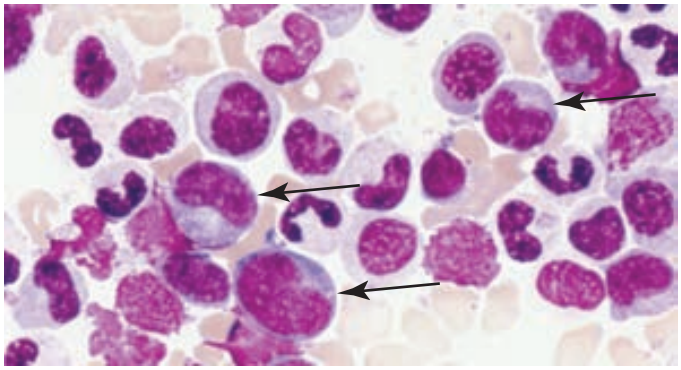
folic acid therapy. Giant, multinucleated erythroid precursors can be found (Figure 25-7 ■). Other nuclear abnormalities include fragmentation, abnormal shape, budding, karryohexis, and irregular staining properties. The cytoplasm of erythroid precursors can show defective hemoglobinization, vacuoles, and basophilic stippling. The presence of ring sideroblasts, reflecting the abnormal erythrocyte metabolism, is a common finding. The International Working Group on Morphology of MDS (IWGM-MDS) defines **ring sideroblasts** as erythroblasts in which at least five mitochondrial iron deposits encircle at least one-third or more of the circumference of the nucleus.[27]

Dysgranulopoiesis

Granulopoiesis is usually normal to increased in patients with MDS unless the overall marrow is hypocellular. Abnormal granulocyte maturation (dysgranulopoiesis), however, is almost always present (Figure 25-8 ■). One of the major findings of dysgranulopoiesis in the bone marrow is abnormal staining of the primary granules in promyelocytes and myelocytes. Sometimes the granules are larger than normal, and at other times are absent. Secondary granules can be



■ **FIGURE 25-7** Arrow points to a multinucleated polychromatophilic erythroblast in the bone marrow of a patient with MDS. It is also megaloblastoid (Wright-Giemsa stain, 1000× magnification).



■ **FIGURE 25-8** Bone marrow from a patient with MDS. Note the metamyelocytes (arrows) and bands with a lack of secondary granules. The bands have light bluish-pink cytoplasm, and the metamyelocytes have a darker bluish cytoplasm (Wright-Giemsa stain, 1000× magnification).

absent in myelocytes and other more mature neutrophils, giving rise to the hypogranular peripheral blood neutrophils. Irregular cytoplasmic basophilia with a dense rim of peripheral basophilia is also characteristic. Nuclear abnormalities similar to those found in the peripheral blood granulocytes can be present in bone marrow granulocytes.

Dysmegakaryopoiesis

Megakaryocytes can be decreased, normal, or increased. The presence of micromegakaryocytes, large mononuclear megakaryocytes, and megakaryocytes with other abnormal nuclear configurations (Figure 25-9 ■) reflect abnormalities in maturation. Megakaryocyte nuclei can be hyperlobulated or hypolobulated and can show multiple widely separated nuclei. The lack of granules or presence of giant abnormal granules is also characteristic.

Dysplastic micromegakaryocytes with a single eccentric nucleus are common in del(5q) and in cases with abnormalities of chromosome 3.[3]

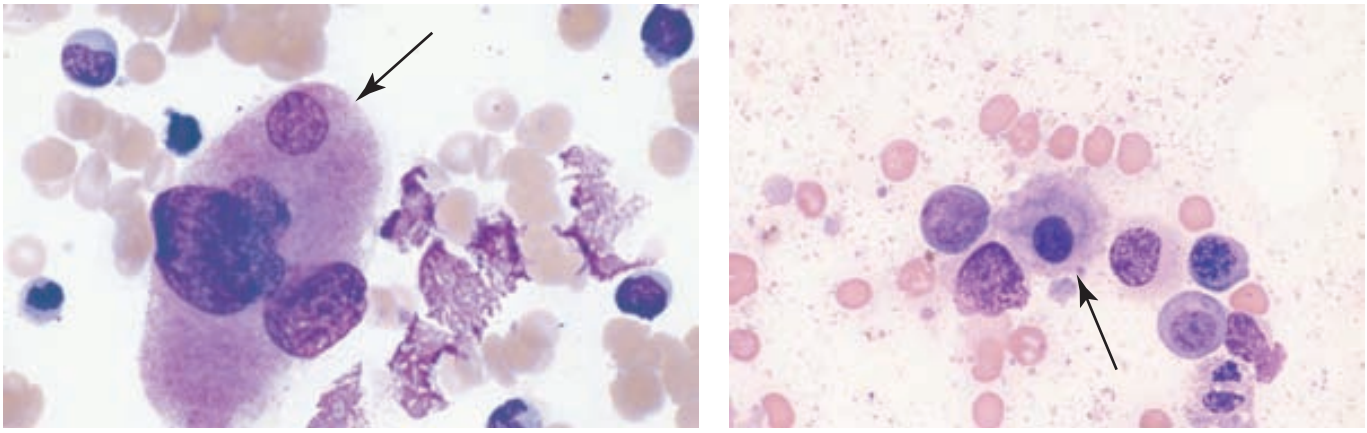
Molecular Diagnostics

Although there are no chromosomal abnormalities that allow immediate and exclusive diagnosis of MDS, genomic instability figures importantly in the development of myelodysplastic syndromes.[8,9,28] Cytogenetic studies are useful because an abnormal karyotype can help diagnose ambiguous cases of MDS, and specific karyotypes can influence the prognosis.

A fluorescence in situ hybridization (FISH) panel can provide diagnostic and prognostic information for MDS and can be used to monitor response to therapy or progression of the disease (Chapter 42). Specific FISH probes should identify the commonly encountered abnormal karyotypes. Rapidly developing molecular diagnostic testing will undoubtedly include gene expression analysis by microarray, next generation sequencing, and whole genome analysis to elucidate the complicated genetic profile of myelodysplastic syndromes.[8,29]

Other Laboratory Findings

Serum iron and ferritin levels are normal or increased, and the total iron-binding capacity (TIBC) is normal or decreased, distinguishing MDS from iron-deficiency anemia (IDA). Cobalamin



a b

■ **FIGURE 25-9** **(a)** Abnormal megakaryocyte (arrow) from a patient with MDS. **(b)** Arrow points to a micromegakaryocyte in the bone marrow of a patient with MDS. Note the megaloblastoid features of the surrounding cells (both are bone marrow; Wright-Giemsa stain, 1000× magnification).

(vitamin B_{12}) and folic acid levels are normal to increased, a feature that helps to differentiate MDS with megaloblastoid features from megaloblastic anemias with the typical megaloblastic features. Lactic dehydrogenase (LD) and uric acid levels can be increased as a result of ineffective hematopoiesis.[19]

☑ CHECKPOINT 25-4

Why is serum cobalamin serum folate level or bone marrow iron stain important in diagnosing MDS?

CASE STUDY (continued from page 485)

A bone marrow was performed on Hancock. The marrow showed a cellularity of about 75%. There was myeloid hyperplasia with 9% blasts, 26% promyelocytes, 18% myelocytes, 6% metamyelocytes, 4% bands, and 37% eosinophils. The ratio of myeloid-to-erythroid precursors (M:E) was 12:1. The myelocytes were hypogranular, and some had two nuclei. The erythroid precursors showed megaloblastoid changes. Megakaryocytes were adequate in number but showed abnormal forms with nuclear separation and single nucleated forms.

6. Which of the hematopoietic cell lines exhibit dyshematopoiesis in the bone marrow?
7. How would you classify the bone marrow cellularity?
8. What does the M:E ratio indicate?
9. Identify at least two features of the bone marrow that are compatible with a diagnosis of MDS.
10. What chemistry tests would be helpful to rule out megaloblastic anemia?

BLAST AND PRECURSOR CELL CLASSIFICATION

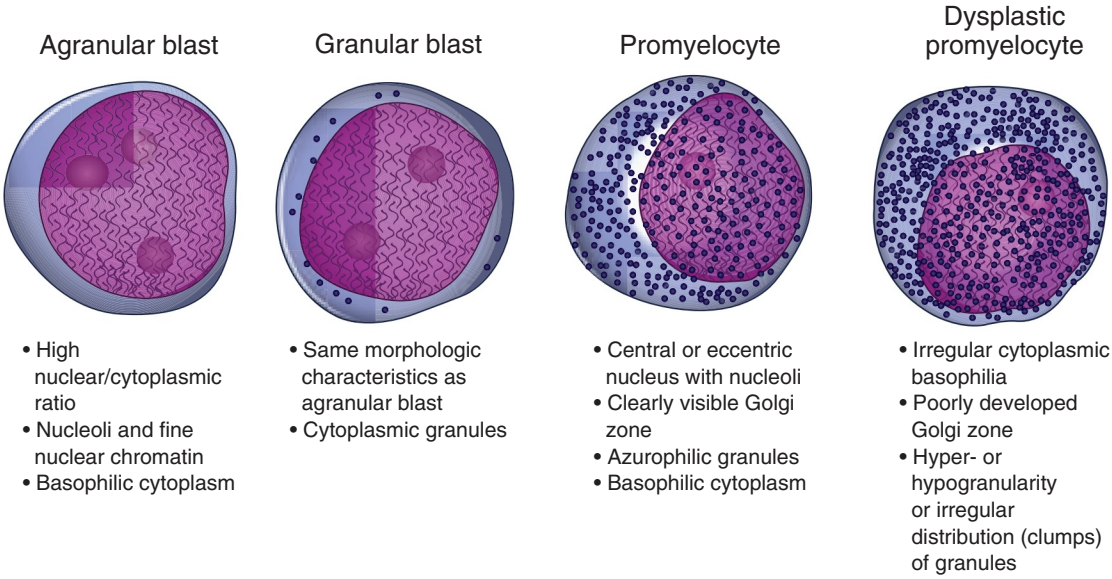
For patients with MDS, the blast count appears to be the most important prognostic indicator of survival and progression to acute leukemia. The maximum number of blasts compatible with a diagnosis of MDS is 19%, whereas the minimum criterion for a diagnosis of acute leukemia is at least 20% blasts. Correctly identifying blasts and type and degree of dysplasia is important in MDS because these characteristics are the basis of classifying subtypes and differentiating them from AML. A 200-cell differential should be performed on peripheral blood, and at least 500 cells should be identified in the bone marrow.[30] In 2005 and 2006, IWGM-MDS developed new diagnostic criteria for MDS and redefined MDS myeloblast morphology. In addition to redefining myeloblast morphology, it proposed criteria necessary to define normal and dysplastic promyelocytes and ring sideroblasts. The morphological categories of early cells in MDS established by the IWGM-MDS are granular myeloblasts, agranular myeloblasts, normal promyelocytes, dysplastic promyelocytes, and ring sideroblasts[27] (Figure 25-10 ■).

Myeloblasts

Myeloblasts are defined as either granular or agranular. Granular blasts possess azurophilic granules and can exhibit Auer rods, although this is an unusual finding. Granular blasts include the former type II and type III blasts, but today there is no differentiation of blasts based on the number of granules observed in the cytoplasm. Agranular blasts correspond to the earlier type I classification of MDS blasts. Whether granular or agranular, the blasts of MDS should exhibit visible nucleoli, fine nuclear chromatin, scant basophilic cytoplasm, and, very importantly, should lack a Golgi zone[27] (Figure 25-11 ■).

Promyelocytes

Differentiating between granular blasts and promyelocytes is essential. Promyelocytes present with most of the features of granular blasts, including fine azurophilic cytoplasmic granules. The IWGM-MDS



■ **FIGURE 25-10** Blasts and promyelocytes as defined by the IWGM-MDS.

has determined that the distinguishing feature between the two cells is the presence of a distinct nonstaining, perinuclear Golgi zone seen in promyelocytes. Promyelocytes of MDS can exhibit dysplasia, which can complicate the morphologic differentiation of granular blasts and promyelocytes. Dysplastic promyelocytes differ from normal promyelocytes in that their chromatin can vary from fine and lacy to coarse, their azurophilic granules can be of variable quantity and distribution, their cytoplasm can stain irregularly, and their Golgi zone may be only faintly visible.[27]

Ring Sideroblasts

The IWGM-MDS has defined three types of sideroblasts, types 1, 2, and 3.

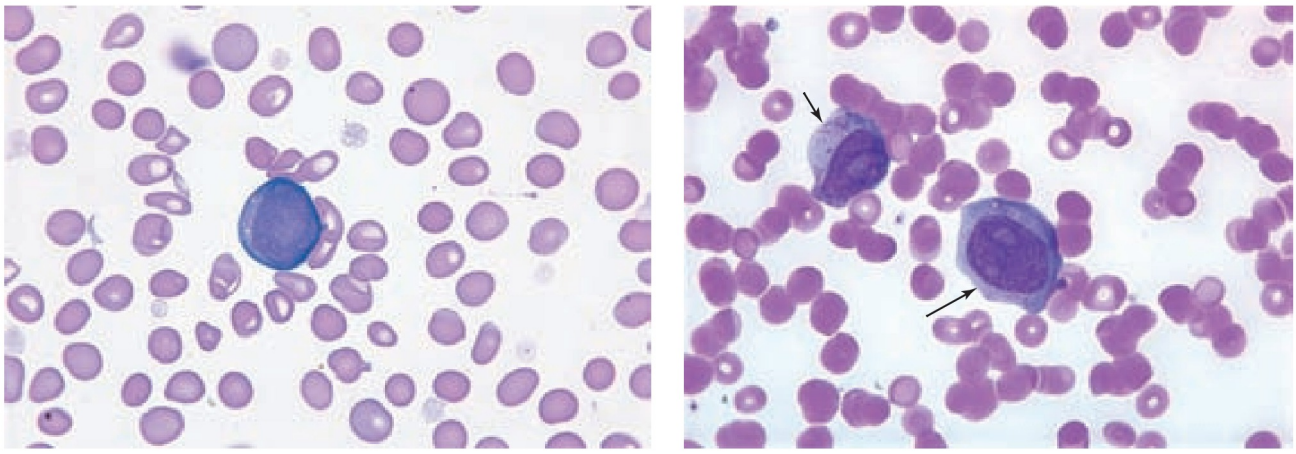
Type 1 sideroblasts are defined as possessing fewer than five siderotic cytoplasmic granules.

Type 2 sideroblasts have five or more siderotic granules, but they lack perinuclear distribution. Type 3 sideroblasts exhibit five or more siderotic granules arranged around the nucleus of the erythroblast so that they encircle at least one-third of the nuclear perimeter.

The IWGM-MDS recommends that a minimum of 100 nucleated erythrocytes at all stages of maturation be included in the count. Note in the section "Classification" that the WHO still defines the required number of ring sideroblasts as 15%.[27]

☑ CHECKPOINT 25-5

Why is it important to correctly identify the number of blasts when evaluating the peripheral blood or bone marrow smear of a patient suspected of having MDS?



a b

■ **FIGURE 25-11** **(a)** Agranular myeloblast. **(b)** Granular myeloblast (arrows) (both: peripheral blood, Wright-Giemsa stain, 1000× magnification).

Cytochemical and Immunological Identification of Blasts

Although the blast cells in MDS are derived primarily from myeloid or monocytic precursors, a panel of cytochemical and immunocytochemical reactions can be performed and interpreted to enhance the accuracy of diagnosis (Chapters 23, 37, 40). Cytochemical stains help to identify the origin of atypical and bizarre dyspoietic cells often seen in MDS.[23] Peroxidase and Sudan black B identify blasts with a myeloid origin; however, the blasts in MDS can have lower peroxidase activity than normal blasts. Combined esterase stains can be performed on both peripheral blood and bone marrow for a more accurate assessment of monocytic cells. Iron stain can reveal abnormal iron metabolism in erythroblasts with the presence of increased iron stores and ring sideroblasts. The presence of blocks of periodic acid-Schiff (PAS)–positive material in erythrocyte precursors indicates abnormal carbohydrate metabolism. Abnormal small megakaryoblasts can be difficult to distinguish from lymphoblasts or agranular myeloblasts. They can be readily identified, however, by immunochemistry with antibodies against platelet-specific glycoproteins IIb/IIIa (CD41), GPIIIa (CD61), or by antibody against factor VIII in histiologic sections. Diaminobenzidine can be utilized to identify platelet peroxidase in electron micrographs.[23]

Immunophenotyping is increasingly used to aid in diagnosis and prognosis of MDS patients.[31] Immunophenotyping is most useful when marrow morphology and cytogenetics are inconclusive and to distinguish MDS cases with a hypoplastic bone marrow from other bone marrow failure disorders, such as aplastic anemia.[22] Aberrant antigenicity observed in MDS includes lymphoid antigens on myeloid cells, over- or underexpression of expected antigens, the presence of immature antigens on mature cells, and expression of mature antigens on immature cells (Table 25-3 ★).[32,33]

CLASSIFICATION

In 1982, the French-American-British (FAB) group proposed a classification scheme for the myelodysplastic syndromes.[34] This classification defined five subgroups based on morphological characteristics, such as the blast count and degree of dyspoiesis in the peripheral blood and bone marrow.

Although the FAB classification system was the benchmark for diagnosing MDS for the past two decades, it did not incorporate the newer diagnostic technologies such as cytogenetics and immunophenotyping.[20,35] In 2001, the WHO published a new classification system developed by a group of American and European pathologists, hematologists, and oncologists. This new system incorporated morphology, immunophenotype, and genetics with clinical and prognostic features into a classification of all neoplastic diseases of the hematopoietic and lymphoid tissues (Chapter 23). This resulted in significant changes in the classification of MDS and was revised again in 2008. The WHO 2008 Classification System incorporates new scientific and clinical information to improve the diagnosis of the previously recognized categories and to introduce newly identified disease categories.[30]

The 2008 WHO Classification System criteria for each subgroup of MDS are shown in Table 25-4 ★. A major change in the 2008 revision is the creation of a new category that replaces "refractive anemia" and includes the other types of unilineage dysplasia. This new category, *refractive cytopenia with unilineage dysplasia (RCUD)*, includes cases with 10% or more dysplastic cells in a single lineage, either refractory anemia, neutropenia, or thrombocytopenia. The revised criteria also redefine refractory anemia with excess blasts (RAEB-1) to include cases that consistently show 2–4% blasts in the peripheral blood even if the blast percentage in the bone marrow is <5%. The category *refractory cytopenia with multilineage dysplasia (RCMD)* is no longer subdivided based on the percentage of ringed sideroblasts in the bone marrow. A provisional category, *refractory cytopenia of childhood (RCC)*, was added to include children with cytopenia(s) with <2% blasts in the peripheral blood, <5% blasts in the bone marrow, and evidence of dysplasia in two or more lineages. Provisional categories are newly described diseases for which additional studies are needed to clarify their significance.[30] Studies to evaluate the clinical usefulness of the WHO classification conclude that it clearly identifies more homogenous subgroups and enables clinicians to select the best treatments and better predict prognosis and clinical responses.[20,23] At this time, most clinicians have adopted the 2008 WHO Classification System, and it is followed in this chapter.

★ **TABLE 25-3** Characteristic Flow Cytometric Analysis of Myelodysplastic Syndromes

- Reduced granulocytic side scatter due to hypogranularity of granulocyte cytoplasm
- Decreased CD15, CD10, CD16, CD11b, and CD13 expression on myeloid progenitors and maturing myeloid cells
- Loss of synchronized expression on granulocytes including the failure of CD64/CD33, CD16/CD13, and CD16/CD11b antigens to co-express as expected
- Characteristic lymphoid antigens such as CD2, CD5, CD7, CD19, and CD56 inappropriately expressed on granulocytes and/or monocytes
- Decreased or absent CD4, CD13, and HLA-DR antigen expression on monocytes
- Retention of an abnormally immature phenotype due to expansion of immature CD34+ and CD34−/CD117+ cells

DESCRIPTION OF SUBGROUPS OF MDS

The 2008 WHO Classification System identifies six MDS subgroups:

1. Refractory cytopenia with unlineage dysplasia (RCUD)
   - Refractory anemia
   - Refractory neutropenia
   - Refractory thrombocytopenia
2. Refractory anemia with ring sideroblasts (RARS)
3. Refractory cytopenia with multilineage dysplasia (RCMD)
4. Refractory anemia with excess blasts (RAEB)
   - RAEB-1
   - RAEB-2
5. MDS associated with isolated del(5q)
6. Myelodysplastic syndrome, unclassifiable (MDS, U)

★ **TABLE 25-4** 2008 World Health Organization Classification Criteria for Myelodysplastic Syndromes

Classification	Peripheral Blood	Bone Marrow
Refractory cytopenia with unilineage dysplasia (RCUD)	Unicytopenia or bicytopenia[a]	Unilineage dysplasia
	Rare or no blasts (<1%)	≥10% of cells in affected lineage are dysplastic
Refractory anemia (RA)		<5% blasts
Refractory neutropenia (RN)		<15% ring sideroblast
Refractory thrombocytopenia (RT)		
Refractory anemia with ring sideroblasts (RARS)	Anemia	≥15% ring sideroblasts
	No blasts	Erythroid dysplasia only
		<5% blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenias (bicytopenia or pancytopenia)	Dysplasia in ≥10% of the cells of two or more lineages
	Rare or no blasts (<1%)	<5% blasts in marrow
	No *Auer* rods	No Auer rods
	$<1 \times 10^9$/L monocytes	Variable % of ring sideroblasts
Refractory anemia with excess blasts 1	Cytopenias	Unilineage or multilineage dysplasia
	<5% blasts	5–9% blasts
	No *Auer* rods	No Auer rods
	$<1 \times 10^9$/L monocytes	
Refractory anemia with excess blasts 2	Cytopenias	Unilineage or multilineage dysplasia
	5–19% blasts	10–19% blasts
	± Auer rods	± Auer rods
	$<1 \times 10^9$/L monocytes	
MDS associated with isolated del(5q)	Anemia	Normal to increased megakaryocytes with hypolobulated nuclei
	Usually normal or increased platelet count	<5% blasts
	No or rare blasts (<1%)	Isolated del(5q) cytogenetic abnormality
		No Auer rods
Myelodysplastic syndrome, unclassifiable	Cytopenias	Unequivocal dysplasia in <10% of cells in 1 or more cell lines
	Rare or no blasts (≤1%)	<5% blasts
		Cytogenetic abnormalities characteristic of MDS

[a]Bicytopenia is occasionally observed. Cases with pancytopenia should be classified as MDS, unclassified.

Zhou J, Orazi A, Czader MB. Myelodysplastic syndromes. *Semin Diag Path*. 2001;28:258–72.

Vardiman JW, Thiele J, Arber DA et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–51.

Brunning RD, Orazi A, Germing U et al. Myelodysplastic syndromes. In Swerdlow SH, Campo E, Harris NL et al., eds. *World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Hematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press, 2008:89–93.

Refractory Cytopenia with Unilineage Dysplasia (RCUD)

The category **refractory cytopenia with unilineage dysplasia (RCUD)** is for cases with isolated cytopenia or bicytopenia accompanied by unilineage dysplasia. It includes refractory anemia (RA) and less common cases of refractory neutropenia and refractory thrombocytopenia. These cases together make up approximately 10–20% of all types of MDS; refractory neutropenia and refractory thrombocytopenia represent only 1–2%. In general, RCUD cases have an extended course with the rate of progression to AML of <5% at 5 years. Most patients present with a normocytic, normochomic, or sometimes macrocytic anemia. Variation in size and shape of the red blood cells is common. Less than 1% blasts are present in the peripheral blood and <5% blasts are in the bone marrow. The bone marrow is hypercellular because of increased erythropoiesis or sometimes normocellular. Dyserythropoiesis is present without prominent ring sideroblasts. In RA, dysplasia is seen in <1–2% in the granulocytic and thrombocytic cells.

Chromosomal analysis is frequently helpful in diagnosing RCUD because of the minimal morphologic changes in some cases. Excluding other reactive causes of cytopenias and associated dysplasia is also important. Patients with pancytopenia and morphologic dysplasia of only one cell line should be classified as MDS, unclassified (MDS, U) rather than this category.

Refractory Anemia with Ring Sideroblasts (RARS)

Refractory anemia with ring sideroblasts (RARS) presents as an unexplained anemia with morphologic dysplasia in the erythroid cell line. Ring sideroblasts account for ≥15% of the erythroid precursor cells in the bone marrow. This subgroup accounts for approximately 5–10% of all cases of MDS. The anemia is usually macrocytic and is less often normocytic. Sometimes evidence of a dual population of normochromic and hypochromic cells exists. The peripheral blood shows reticulocytopenia and often leukopenia. The platelet count is occasionally increased. A few cases exhibit granulocyte and platelet morphologic abnormalities; however, classic cases of RARS have no significant dysplasia in these cell lines.

The bone marrow is hypercellular with megaloblastoid dyserythropoiesis. If dysgranulopoiesis and dysmegakaryopoiesis are present, they are mild. The marrow has <5% blast cells. Chromosomal abnormalities appear in < 10% of cases. Approximately 1–2% of cases evolve into AML. Secondary causes of ring sideroblasts, such as exposure to toxic substances, certain drugs, and alcoholism should be ruled out.[24]

Refractory Cytopenia with Multilineage Dysplasia (RCMD)

Refractory cytopenia with multilineage dysplasia (RCMD) is characterized by dysplastic features in at least 10% of the cells in two or more cell lineages, <5% blasts in the bone marrow, and <1% blasts in the peripheral blood. This is one of the most common subgroups, representing 30–40% of all MDS cases. Studies have shown that individuals with RCMD have a worse prognosis than those with dysplasia in only one lineage; thus, a new subgroup was added in 2001.[20] Patients usually display cytopenias in two or more lineages and have $<1 \times 10^9$/L monocytes present in the peripheral blood. Ring sideroblasts can exceed 15% of the nucleated red blood cells in the marrow. Chromosomal abnormalities can be present in up to 50% of cases in this category, some being complex. The frequency of evolution to AML is approximately 10% within 2 years of onset.

Refractory Anemia with Excess Blasts (RAEBs)

In **refractory anemia with excess blasts (RAEBs)**, cytopenia occurs in at least two lineages and conspicuous qualitative abnormalities are in all three lineages. The anemia is normocytic or slightly macrocytic with reticulocytopenia. Evidence of dysgranulopoiesis is prominent. The peripheral blood has 2–19% blasts. Monocytosis without leukocytosis can be present, but the absolute monocyte count does not exceed 1×10^9/L, and serum and urinary lysozyme levels are normal. Platelet abnormalities include giant forms, abnormal granularity, and functional aberrations. Sometimes circulating micromegakaryocytes can be found.

The bone marrow is hypercellular but less often is normocellular with varying degrees of granulocytic and erythrocytic hyperplasia. All three lineages show signs of dyshematopoiesis. ALIP is most common in this subtype.[3] The proportion of blasts varies from 5–19%. Abnormal promyelocytes can be present. These abnormal cells have blastlike nuclei with nucleoli and no chromatin condensation, and the cytoplasm contains large bizarre granules. Ring sideroblasts may be increased, but the elevated blast count differentiates RAEB from RARS. In some cases, differentiating RAEB from acute leukemia is difficult. Serial examinations are sometimes necessary to make an accurate diagnosis.

A difference in survival for patients having 5–9% blasts and those having 10–19% blasts has been shown. Patients with >9% blasts have a worse outcome. The separation of these two groups into RAEB-1 (5–9% blasts in bone marrow, <5% blasts in the peripheral blood) and RAEB-2 (10–19% blasts in the bone marrow, 5–19% blasts in the peripheral blood) provides a more accurate prognostic classification.[15] If the blast percentage is <5% in the bone marrow and 2–4% blasts are consistently present in the blood, these cases are also classified as RAEB-1. Cases with <5% blasts in the peripheral blood and <10% blasts in the bone marrow and Auer rods present are also placed in RAEB-2.[24]

This subgroup is one of the most common, accounting for 30–40% of MDS cases and has the worst prognosis. Approximately 25% of RAEB-1 and 35–50% of RAEB-2 progress to AML.[3]

MDS Associated with Isolated del(5q)

MDS patients with an isolated deletion of the long arm of chromosome 5 (del[5q] or 5q−) and no other chromosomal abnormalities appear to have a unique disease course characterized by a favorable prognosis and low risk of transformation into AML. Women have a marked predominance of cases (70%), and the mean age at presentation is 66 years. The main features are macrocytic anemia, moderate leukopenia, normal to increased platelet count, hypolobulated megakaryocytes, and <1% blasts in the peripheral blood and <5% in the bone marrow. The bone marrow is usually hypercellular or normocellular with normal to increased megakaryocytes, some with hyperlobulated nuclei.[24] The *JAK2* V617F mutation has recently been found in a small subset of patients with isolated del(5q). These patients have slightly higher WBC and platelet counts but no separate classification has been established to date.[3]

Myelodysplastic Syndrome, Unclassifiable

When the MDS does not fit any of the defined WHO subgroups, it is placed in the MDS, U category. Examples are cases of RCUD or RCMD with 1% myeloblasts in the blood, MDS with pancytopenia and dysplasia limited to one cell lineage, or patients with no increase in blasts, persistent cytopenias, and <10% dysplastic cells in any lineage but having cytogenetic abnormalities consistent with MDS.[3,30]

Childhood MDS

Childhood MDS is rare and accounts for <5% of cases of hematopoietic neoplasms in children. The manifestation of MDS in adults and in children has major differences, and pediatric hematologists deemed previous classification systems not useful.[36]

The 2008 WHO classification of MDS introduced a provisional entity, refractory cytopenia of childhood (RCC), specifically for childhood cases of MDS with <2% blasts in the peripheral blood and <5% in the marrow and persistent cytopenias associated with dysplasia in at

least two cell lines. Other childhood cases of MDS with 2–19% blasts in the peripheral blood and 5–19% blasts in the bone marrow should be categorized the same as for adults. Dysplasia in children with MDS is less pronounced, and the more aggressive subtype (RAEB) predominates; progression to acute leukemia is faster than in adults. Age of 2 years or less and a hemoglobin F level of 10% or higher are associated with a poor prognosis.[22] Cytogenetic abnormalities are seen in approximately 70% of cases, and monosomy 7 is the most common cytogenetic change. Unlike adults, abnormalities of chromosome 7 do not seem to be associated with a poor prognosis in children. One-third of children with MDS have genetic predisposition syndromes, such as Down syndrome.[22] These cases have been moved to a new category, myeloid leukemia in Down syndrome, and are now excluded from the MDS.[36]

☑ CHECKPOINT 25-6

When a diagnosis of MDS is considered for a patient, why must other causes of reactive cytopenias be ruled out? What type of testing helps determine the diagnosis of MDS?

CASE STUDY *(continued from page 487)*

Review Hancock's peripheral blood and bone marrow features previously identified.

11. What is the most likely MDS subgroup, and on what criteria is the answer based?

VARIANTS OF MDS

A number of patients have blood and/or marrow findings that cause diagnosis and/or classification problems. Some of these occur often enough to consider them as variants of MDS, but the 2008 WHO classification does not formally recognize them. The recommendation is that these cases be classified according to one of the subgroups and note the unusual morphologic features, such as "RAEB-2 with hypoplastic marrow."[3,24]

Hypoplastic MDS

Although most MDS cases are associated with hypercellular or normocellular bone marrows, about 5–10% have hypocellular marrows. The hypocellular marrow does not appear to affect survival rate, nor does it influence progression to AML.[3] In these cases, bone marrow biopsy is necessary to exclude a diagnosis of aplastic anemia or hypoplastic AML. This distinction is important because the diagnosis influences treatment and prognosis.

Hypoplastic MDS (h-MDS) should be considered when the bone marrow cellularity is <30% or <20% in patients older than 60 years of age. The criteria for MDS must be met in hypoplastic cases as well as in the hypercellular or normocellular cases. Dysplasia can be difficult to identify, and dyserythropoiesis has been described in aplastic anemia. Dysmegakaryopoiesis and dysgranulopoiesis, however, are most characteristic of MDS and can be helpful findings. In addition, ALIP, indicating abnormal bone marrow architecture, is typical of MDS. If present, chromosomal abnormalities help distinguish MDS from aplastic anemia as do immunohistochemistry stains and immunophenotyping by flow cytometry for CD34 and the presence of megakaryocytes.[3] The distinction of MDS from AML can be made based on the blast count. A count >19% indicates AML. Although the pathophysiology of hypoplastic MDS is unknown, secretion of inhibitory cytokines by autoreactive or clonal-involved T cells is believed to suppress normal hematopoiesis.[37]

MDS with Fibrosis

Mild to moderate fibrosis has been described in up to 50% of patients with MDS; marked fibrosis can be seen in 10–15% of cases.[25,38] The incidence of fibrosis appears to be even higher in therapy-related MDS. If fibrosis is present, other diagnoses including primary myelofibrosis (Chapter 24), chronic myelogenous leukemia (Chapter 24), and acute megakaryoblastic leukemia (Chapter 26) should be considered and excluded. MDS patients with fibrosis typically have pancytopenia, excess blasts, hypocellular bone marrow with fibrosis, trilineage dysplasia, small megakaryocytes with hypolobulated nuclei, and no hepatomegaly or prominent splenomegaly.[25,37] The increased fibrosis is thought to be produced by the liberation of cytokines such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) from dysplastic megakaryocytes.[19,25,38]

Therapy-Related Myelodysplasia

Myelodysplasia secondary to alkylating chemotherapy and/or radiotherapy for other malignant or nonmalignant diseases is frequently referred to as *therapy-related* or *treatment-related MDS* (*t-MDS*). It should be noted, however, that MDS can develop as a second primary disorder unrelated to therapy, especially if MDS develops a very short or a very long time after therapy. In the 2008 WHO classification, these cases are placed in the category therapy-related myeloid neoplasms under acute myeloid leukemia and related neoplasms.[39]

Development of MDS or acute leukemia (AL) appears to be related to the therapy's duration, amount, and repetition as well as the patient's age. The highest incidence of t-MDS occurs 3–8 years after treatment, and long-term use of DNA alkylating agents appears to present the highest risk.[23,36] Currently, there is no way to predict which patients will develop MDS after treatment; therefore, monitoring after therapy is not recommended.[23]

Compared with primary MDS, t-MDS tends to have a younger age of onset, an increased frequency and severity of thrombocytopenia, and a higher percentage of patients presenting with RAEB.[40]

In most cases, the qualitative changes are marked with trilineage involvement, typical of RAEB. The number of blasts, however, is usually <5%, typical of refractory anemia. Despite the low percentage of blasts, the clinical course of the disease reflects profound marrow failure, and the outcome is very unfavorable (a median survival of 4–6 months).[40] Complex karyotypes are present in the majority of t-MDS cases.

PROGNOSIS

The median survival for all types of MDS is <2 years; however, some patients can survive many years with continuous transfusion therapy. The mortality rate varies from 58–72%. Leukemic transformation ranges in incidence from 10–40%. The likelihood of transformation to AML increases with the presence of severe cytopenias, more overt dysplastic features of cells, and complex chromosome abnormalities.[19]

The International Prognostic Scoring System (IPSS)[41,42] was developed in 1997 to assist physicians in predicting prognosis and selection of optimal therapy for individuals diagnosed with MDS. This system was developed using data from seven large studies that had previously generated prognostic systems and was accepted worldwide. The prognostic score was based on the percentage of blasts in the bone marrow, cytogenetic abnormalities, and number of cytopenias, and patients were divided into four risk groups with distinct risks of death and leukemic transformation. Low scores indicate prolonged survival.

The WHO Classification-Based Prognostic Scoring System (WPSS) was published in 2011 (Table 25-5 ★).[43] This prognostic scoring system utilizes the WHO category of MDS classification, karyotype, and transfusion requirements and assigns patients to five risk groups of survival and probability of leukemic progression.

In 2012 a revised International Prognostic Scoring System (IPSS-R) was published that integrated additional clinical features to improve prognostic assessment[44] (Table 25-6 ★). The IPSS-R incorporated additional new cytogenetics and clinical features including the degree of cytopenia of three lineages (erythroid, platelets, and neutrophils). The karyotypes were split into five cytogenetic prognostic subgroups, rather than the three subgroups used in the original IPSS, and the marrow blast percentage values were adjusted. It was further refined by categorizing patients into five rather than four prognostic categories. The IPSS-R is based on a much larger combined database

★ **TABLE 25-5** WHO Classification-Based Prognostic Scoring System (WPSS) of Myelodydysplastic Syndromes

Points	WHO Category	Karyotypea[a]	Transfusion requirement[b]
0	RA, RARS, 5q−	Good	None
1	RCMD	Intermediate	Regular
2	RAEB-1	Poor	—
3	RAEB-2	—	—

Use summed points to determine risk category

WPSS Risk Group	Score
Very low	0
Low	1
Intermediate	2
High	3–4
Very high	5–6

[a]Karyotypes: Good = normal, −Y, del(5q−) only, del(20q−) only. Poor = complex (≥3 abnormalities), or chromosome 7 abnormalities. Intermediate = all others.

[b]Transfusion dependency is defined as at least one red blood cell transfusion every 8 weeks over a period of 4 months.

★ **TABLE 25-6** International Prognostic Scoring System-Revised (IPSS-R) for the Myelodysplastic Syndromes

Prognostic Variable	0	0.5	1	1.5	2	3	4
Marrow blasts %	≤2		>2−<5		5–10	>10	
Karyotype[a]	Very Good		Good		Intermediate	Poor	Very Poor
Hemoglobin (g/dL)	≥10		8–10	<8			
Platelets ($\times 10^9$/L)	≥100	50−<100	<50				
Absolute neutrophil count ($\times 10^9$/L)	≥0.8	<0.8					

IPSS Risk Group	Score
Very low	≤1.5
Low	>1.5−3
Intermediate	>3−4.5
High	>4.5−6
Very high	>6

[a]Cytogenetic prognostic subgroups include: Very Good [–Y, del(11q)]; Good [normal, del(5q), del(12p), del(20q), double including del(5q)]; Intermediate [del(7q), +8, +19, i(17q), any other single or double independent clones]; Poor [–7, inv(3)/t(3q)/del(3q), double including –7/del(7q), complex: 3 abnormalities]; Very Poor (complex: >3 abnormalities).

of patients provided by the International Working Group for Prognosis in MDS (IWG-PM) and is expected to provide an improved prognostic categorization model.

CASE STUDY *(continued from page 492)*

Hancock's karyotope showed multiple complex abnormalities.

12. Using the IPSS, what is the prognosis for Hancock?

THERAPY

Myelodysplastic syndromes are so heterogeneous that therapy must be based on the patient's risk stratification, transfusion needs, the number of blasts seen, and more recently, the patient's cytogenetic profile. Patients evaluated as lower risk can be given RBC transfusions to treat anemia associated with MDS. Erythropoiesis-stimulating agents and thrombopoietic receptor agonists may be effective in countering thrombocytopenia.[45] Antibiotics are necessary for infections that are likely to follow neutropenia but are not recommended as a prophylactic treatment.[46] The National Comprehensive Cancer Network (NCCN) recommends the use of iron chelation therapy in patients with ferritin levels ≥2500 mg/L.[45,47]

Immunosuppressive therapy such as that with cyclosporine or antithymocyte globulin (ATG) is controversial but may be considered in some patients. ATG consists of pooled antibodies against human T lymphs. This treatment can be effective because abnormal autoimmune activity of activated T cells against myelocytic cell lines may contribute to the development of MDS.[47,48]

The U.S. Food and Drug Administration (FDA) has approved three drugs for the treatment of MDS: azacitidine, decitabine, and lenalidomide. Azacitidine and its deoxy derivative decitabine are pyrimidine nucleoside analogues of cytidine. As such, they incorporate into DNA to act as false substrates and in this way reversibly inhibit DNA methyltransferase to block DNA methylation. The resulting hypomethylation can activate tumor suppressor genes silenced by hypermethylation, producing an antitumor effect. Although the mechanism of action of lenalidomide remains incompletely understood, it appears to have multiple mechanisms of action including direct antitumor effect, inhibition of the microenvironment that supports tumor cells, and an immunosuppressive function. Interestingly in MDS with del(5q), lenalidomide inhibits the malignant cell line, but in MDS without 5q deletions, lenalidomide exerts its effect by promoting erythropoiesis.[49]

The treatment options for patients with higher risk MDS could include the same treatment options for lower risk patients but traditionally have also utilized classical combination therapy with anthrocycline and cytarabine. The most important prognostic factor governing the response of patients to such AML-like therapy is the patient's karyotype. Patients with −7, del(7q), or complex karyotype do not respond favorably to this regimen.[47] Allogeneic stem cell transplantation is usually not recommended for patients with lower risk MDS but remains a viable treatment option for children and higher risk patients and is potentially curative.[40,47,50]

MYELODYSPLASTIC/ MYELOPROLIFERATIVE NEOPLASMS (MDS/MPNs)

The category of **myelodysplastic/myeloproliferative neoplasms (MDS/MPNs)** includes clonal hematopoietic neoplasms that, at the time of initial presentation, have some clinical, laboratory, or morphologic findings of both an MDS and an MPN.[30,51] Typically, patients with MDS/MPN have a hypercellular bone marrow associated with proliferation in one or more of the myeloid lineages. Frequently, the proliferation is effective and results in increased numbers of circulating cells. However, these cells commonly are morphologically and functionally dysplastic. While one or more lineages can show hyperproliferation, one or more other lineages can exhibit ineffective proliferation and resulting cytopenia(s). The percentage of blasts in the bone marrow and peripheral blood is always <20%. At present, there are no identified genetic defects specific for any of the entities included in this category. However, recurring chromosomal and molecular abnormalities have been described (Table 25-7 ★).

Clinical symptoms can result from cytopenia(s) when present, dysplastic cells that do not function properly, and leukemic infiltration of various organs. Splenomegaly and hepatomegaly are commonly found, but the clinical presentation is highly variable. The incidence varies widely, depending on the specific disease, as do the prognosis and tendency for clonal evolution and disease progression. The disorders included in MDS/MPN are:

- Chronic myelomonocytic leukemia
- Atypical chronic myeloid leukemia, *BCR/ABL1*$^{-}$
- Juvenile myelomonocytic leukemia, and
- MDS/MPNs, unclassifiable (Table 25-7).

Chronic Myelomonocytic Leukemia

Chronic myelomonocytic leukemia (CMML) is a clonal hematopoietic neoplasm associated with a persistent monocytosis (>1 × 10^9/L) in the peripheral blood (Figure 25-12 ■). Although the finding of absolute monocytosis is required for diagnosis, other hematologic findings are remarkably variable. Leukocytosis, leukopenia, neutrophilia, and neutropenia are all possibilities in CMML. Dyshematopoiesis can range from minimal expression in a single lineage to marked dysplasia in all the lineages.

The cell of origin is believed to be the HSC. Clonal cytogenetic abnormalities are found in 20–40% of patients, but none are specific. The most frequently reported abnormalities include +8, −7/del(7q), del(12p), del(20q), isochromosome 17q [i(17q)], and complex karyotype. Mutations of the *N-RAS/K-RAS*, *RUNX1*, *TET2*, *CBL*, or *ASX1* proto-oncogenes are found in ~40% of patients.[52]

With an annual incidence of ~3/100,000, most patients with CMML are older than 50 years (median age at diagnosis ~70 years). Median survival time is 20–40 months, and progression to acute leukemia is seen in 15–30% of cases.

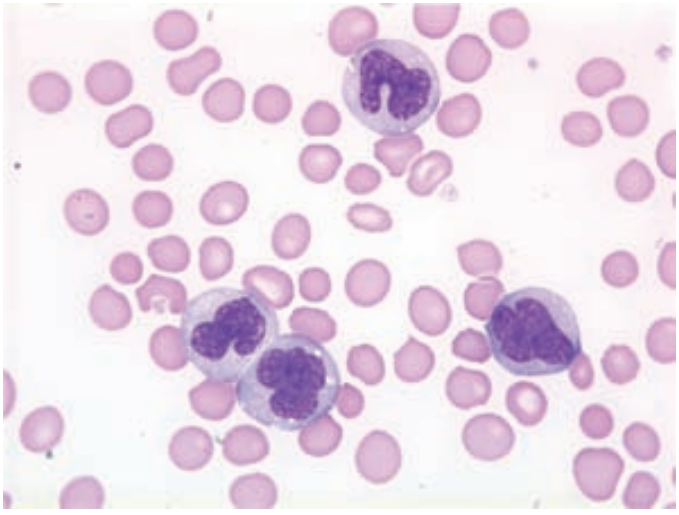
The WHO recommendations of diagnostic criteria for CMML are as follows: peripheral blood monocytosis >1 × 10^9/L, <20% blasts (myeloblasts, monoblasts, and promonocytes) in the peripheral blood and bone marrow, dysplasia in one or more myeloid lineages,

★ **TABLE 25-7** The Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPNs)

Classification	Peripheral Blood	Bone Marrow	Genetics	Immunophenotype
Chronic myelomonocytic leukemia (*CMML*-1)	Monocytosis $>1 \times 10^9$/L; <5% blasts (including promonocytes)	<10% blasts (including promonocytes); dysplasia of ≥1 myeloid lineages	*BCR/ABL1*−	CD33, CD13+; CD14, CD68, CD64 variable; lysozyme+
CMML-2	5–19% blasts (including promonocytes)	10–19% blasts (including promonocytes or when Auer rods present, regardless of blast/promonocyte count	(same as *CMML*-1)	(same as *CMML*-1)
Atypical chronic myeloid leukemia (aCML, *BCR/ABL1*−)	Leukocytosis (<10% monocytes); <5% blasts; promyelocytes, myelocytes, metamyelocytes 10–20%	Hypercellular; <20% blasts; dysgranulopoiesis; occasionally dyserythropoiesis	*BCR/ABL1*−	CD33, CD13+; MPO+
Juvenile myelomonocytic leukemia (JMML)	Monocytosis $>1 \times 10^9$/L; <20% blasts	Hypercellular; <20% blasts; dysgranulopoiesis; occasionally dyserythropoiesis	*BCR/ABL1*−	CD33, CD13+; CD14, CD68, CD64 variable; lysozyme+
Myelodysplastic/ myeloproliferative neoplasm, unclassifiable (MDS/MPN, U)	Anemia, leukocytosis and/ or thrombocytosis; <20% blasts	Hypercellular; proliferation in any or all myeloid lineages	*BCR/ABL1*−	Nondiagnostic

absence of the *BCR/ABL1* fusion gene, and no rearrangement of *PDGFRA* or *PDGFRB* genes. However, the CMML diagnosis includes some important caveats to these diagnostic criteria. One caution is that abnormal monocytes are often seen in both the peripheral blood and the bone marrow but should be excluded from the blast count. A second is that if dysmyelopoiesis is minimal, a CMML diagnosis is still possible if the other characteristics are present and if frequently occurring genetic markers are present in the hematopoietic cells, if the monocytosis has been persistent for at least 3 months, or if other causes of monocytosis have been excluded.[51,52]

Some CMMLs are more myeloproliferative in nature, whereas others display more dysplastic characteristics. Currently, the WHO proposes that CMMLs be divided into two subcategories, depending on the number of blasts present in the peripheral blood and bone marrow. Under these guidelines, *CMML-1* is defined as having <5% blasts in the peripheral blood and <10% blasts in the bone marrow. *CMML-2* is defined as presenting with 5–19% blasts in the blood or 10–19% blasts in the marrow or whenever Auer rods are present.[52]



■ **FIGURE 25-12** Increased monocytes in the peripheral blood from a patient with CMML (Wright stain, 1000× magnification).

Atypical Chronic Myeloid Leukemia (aCML, *BCR/ABL1*⁻)

The latest revision of the WHO classification of myeloid neoplasms renamed atypical chronic myeloid leukemia (aCML) to aCML *BCR/ABL1*⁻ to emphasize that the disorder is not merely an atypical CML. **Atypical chronic myeloid leukemia (aCML, BCR/ABL1⁻)** is a variant of MDS/MPN characterized by primary involvement of the neutrophil series with leukocytosis involving dysplastic immature and mature neutrophils. Multilineage dysplasia is common.

The annual incidence of aCML, *BCR/ABL1*⁻ is ~3 in 100,000. Most patients are older with the median age at diagnosis in the 7th to 8th decade. Median survival time is <20 months, and progression to acute leukemia is seen in 25–40% of cases.

The cell of origin is believed to be the common myeloid progenitor cell (CMP). Reported cytogenetic abnormalities are similar to CMML. They include +8, +13, del(20q), i(17q), and del(12p). The *BCR/ABL1* fusion gene is absent, and the specific genes *N-RAS/K-RAS, RUNX1, TET2, CBL,* or *ASX1* are mutated in nearly 30% of the cases.[52]

For a diagnosis of aCML, *BCR/ABL1*⁻, the peripheral blood WBC must be in excess of 13×10^9/L with increased dysplastic neutrophils and their precursors. Promyelocytes, myelocytes, and metamyelocytes comprise >10% and blasts constitute <20% of the peripheral blood leukocytes. Absolute monocytosis and basophilia are mostly absent. Dysplasia and ineffective hematopoiesis in other cell lines frequently result in anemia and thrombocytopenia.

aCML, *BCR/ABL1*⁻ typically presents with a hypercellular bone marrow. Dysplastic granulocytes account for most of the hypercellularity, dysplasia of the erythrocytic and megakaryocytic lineages may or may not be present. Immunophenotype and cytochemistry results

are typical for the neutrophil lineage although LAP scores can be elevated, normal, or low.

☑ CHECKPOINT 25-7

How do the laboratory findings of aCML, *BCR/ABL1*$^{-}$ differ from those observed in CML (Chapter 24)?

Juvenile Myelomonocytic Leukemia

Juvenile myelomonocytic leukemia (JMML) is an aggressive childhood myeloproliferative disorder characterized by proliferation and immaturity of the granulocytic and monocytic lineages in the peripheral blood and bone marrow. Dysmyelopoiesis and dyserythropoiesis but rarely dysmegakaryopoiesis are evident in the bone marrow. Concomitantly, as a result of the myelomonocytic expansion in the bone marrow, JMML patients often present with anemia and thrombocytopenia.

The cell of origin is believed to be the HSC. Gain of function mutations of *N-RAS* and *K-RAS* and disruption of tumor suppressor genes are essential initiating events in the development of JMML. Moreover, somatic mutations in *PTPN11* (that encodes a protein tyrosine phosphatase) have been found in 35% of JMML cases and have been recommended for inclusion in the diagnostic criteria.[53] Monosomy 7 can also be included for diagnostic purposes.

The annual incidence of JMML is 0.13/100,000 children (age 0–14 years of age). It accounts for <2–3% of all leukemia in children. The age at diagnosis is variable (from 1 month to early adolescence), but 75% of cases are diagnosed in children <3 years old. A significant association of JMML with neurofibromatosis type 1 (NF-1) exists with ~10% of cases of JMML occurring in children with this diagnosis. The overall prognosis is poor, although survival times can vary based on the type of therapy chosen. Without effective treatment, most children die from organ failure as the result of leukemic infiltration. About 10–20% progress to acute leukemia.

The following criteria have been established for the diagnosis of JMML: absolute monocytosis ($>1 \times 10^9$/L), <20% blasts in the peripheral blood and bone marrow, absence of the *BCR/ABL1* fusion gene in addition to at least two of the following: myeloid precursors in the peripheral blood, leukocytosis of $>10 \times 10^9$/L, increased fetal hemoglobin for the patient's demographic, or hypersensitivity of the hematopoietic progenitor to GM-CSF.

Myelodysplastic/Myeloproliferative Neoplasm, Unclassifiable (MDS/MPN, U)

This subcategory, MDS/MPN, U, is used for cases that have clinical, laboratory, and morphologic features that support a diagnosis of both MDS and MPN but do not meet the criteria of the other entities included in the MDS/MPN category. No cytogenetic or molecular genetic findings are specific for this group. The presence of the *BCR/ABL1* fusion gene must always be excluded.

The MDS/MPN, U disorders are characterized by the proliferation of one or more of the myeloid lineages that are ineffective and/or dysplastic. Proliferation of the other myeloid lineages is generally effective with or without dysplasia. Anemia (with or without macrocytosis) usually occurs, occasionally with a dimorphic population of RBCs. The bone marrow is hypercellular and can show proliferation in any or all of the myeloid lineages. Dysplastic features are present in at least one lineage. Typically, the patient has <20% blasts in the blood and bone marrow and prominent myeloproliferative features associated with either thrombocytosis or leukocytosis. The bone marrow and peripheral blood are always involved; extramedullary tissues (liver, spleen) are sometimes also involved.

Summary

The myelodysplastic syndromes are pluripotential hematopoietic stem cell disorders characterized by one or more peripheral blood cytopenias and prominent cellular maturation abnormalities. The bone marrow is usually normocellular or hypercellular, indicating a high degree of ineffective hematopoiesis. The most commonly encountered abnormal karyotypes in MDS are the −5/del(5q), −7/del(7q), +8, del(20q), −Y, and complex karyotype. Mutated genes of significance include *ASXL1*, *EGR1*, *EVI1*, *FLT3*, *JAK2*, *NPM1*, *N-RAS*, *K-RAS*, *RUNX1*, and *p53*.

Although anemia is the most common cytopenia, neutropenia and thrombocytopenia also occur. Erythrocytes are macrocytic or less frequently normocytic. Erythropoiesis in the bone marrow is abnormal with megaloblastoid features commonly present. Neutrophils can show hyposegmentation of the nucleus and hypogranulation. Megakaryocytes also show megaloblastoid features. Platelets can be large and agranular.

The WHO group has classified the MDSs into six subgroups depending on the blast count, degree of dyspoiesis and cytopenias, and presence of abnormal cytogenetic findings. These include RCUD, RARS, RCMD, RAEB, del(5q), and MDS, U. Subgroups with higher blast counts and involvement of multiple lineages in dyspoiesis and complex cytogenetic abnormalities are more aggressive disorders. MDS frequently terminates in acute leukemia; treatment is variable because of the heterogeneous presentation of the disease. Treatment options are made based on multiple clinical parameters including the patient's cytogenetic profile.

The myelodysplastic/myeloproliferative neoplasm category (MDS/MPN) includes disorders that have features of both a myeloproliferative neoplasm and a myelodysplastic syndrome simultaneously. This category includes CMML, aCML, *BCR/ABL1*$^{-}$, JMML, and MDS/MPN, U. An important diagnostic consideration is absence of the *BCR/ABL1* fusion gene in these patients.

Review Questions

Level I

1. In addition to the number of blasts, what other criterion is essential for a diagnosis of RARS? (Objective 2)
   A. at least 15% ring sideroblasts
   B. at least 30% ring sideroblasts
   C. dyshematopoiesis in all three cell lineages
   D. pancytopenia
2. A patient with suspected MDS exhibits anemia, neutropenia, anisocytosis, poikilocytosis, oval macrocytes, Howell-Jolly bodies, hypogranular neutrophils, and a few pseudo–Pelger-Huët cells. The differential shows a few immature granulocytes but no blasts. Which MDS subgroup is most likely? (Objective 2)
   A. RCUD
   B. RARS
   C. RCMD
   D. RAEB
3. The WHO classification system incorporates which of the following for diagnosis: (Objective 2)
   A. the presence of cellular dysplasia
   B. cytogenetics
   C. morphology
   D. all of the above
4. The type of anemia usually seen in MDS is: (Objectives 1, 3)
   A. macrocytic, normochromic
   B. normocytic, normochromic
   C. microcytic, hypochromic
   D. normocytic, hypochromic
5. The most common cytopenia(s) seen in MDS is(are): (Objective 3)
   A. leukopenia
   B. thrombocytopenia
   C. anemia
   D. a combination of two of the above
6. The typical bone marrow cellularity in MDS is: (Objective 3)
   A. hypocellular
   B. normocellular
   C. hypercellular
   D. fibrotic
7. aCML, *BCR/ABL1*$^{-}$ is classified as: (Objective 5)
   A. MDS
   B. AML
   C. MPN
   D. MDS/MPN
8. The most common dyserythropoietic finding in the bone marrow in MDS is: (Objective 3)
   A. megaloblastoid development
   B. impaired hemoglobinization
   C. pseudo–Pelger-Huët cells
   D. agranular cytoplasm
9. Which abnormality demonstrates myelocytic dysplasia? (Objective 3)
   A. dimorphism
   B. pseudo–Pelger-Huët cells
   C. sideroblasts
   D. all of the above
10. This group of neoplasms is characterized by a hypercellular bone marrow, effective proliferation of one or more myeloid lineages, but morphologically and functionally dysplastic cells. (Objective 4)
    A. MDS
    B. MPN
    C. MDS/MPN
    D. AML

Level II

1. According to the 2008 WHO Classification System, what is the minimum percentage of bone marrow blasts needed for a diagnosis of acute leukemia? (Objective 4)
   A. 19%
   B. 30%
   C. 5%
   D. 20%
2. The t-MDS differ from primary MDS in that in t-MDS: (Objective 6)
   A. the abnormal stem cell arises de novo
   B. the dysplastic stem cell often develops after chemotherapy
   C. the dysplastic stem cells have fewer and less complex abnormal karyotypes
   D. the prognosis is more favorable

3. Progression of MDS to acute leukemia is characterized by: (Objective 4)
   A. an increase in blast population
   B. decreased bone marrow cellularity
   C. a decreased M:E
   D. splenomegaly
4. The IWGM-MDS has determined that the distinguishing feature between promyelocytes and granular blasts in MDS is: (Objective 2)
   A. the presence of dysplasia in promyelocytes but not in blasts
   B. the presence of a poorly staining golgi in promyelocytes
   C. the presence of fine, azurophilic chromatin in blasts
   D. scant, basophilic cytoplasm in promyelocytes
5. The contrast between a hypercellular bone marrow and a cytopenic peripheral blood film seen in MDS is attributed to: (Objective 1)
   A. premature destruction of abnormal cells in the bone marrow (ineffective hematopoiesis)
   B. production of blood cells outside the bone marrow (extramedullary hematopoiesis)
   C. immune destruction of cells in the peripheral blood
   D. splenic sequestration
6. Which of the following would be most helpful to differentiate RCUD from del(5q)? (Objective 9)
   A. percentage of bone marrow blasts
   B. elevated leukocyte count
   C. presence of nucleated RBCs
   D. karyotype
7. Promising new treatment for low risk MDS includes: (Objective 3)
   A. AML-like chemotherapy
   B. drugs that reverse epigenetic changes in the dysplastic stem cell
   C. hematopoietic growth factors
   D. bone marrow transplant

Use the following case history to answer questions 8–10.

A patient presents with the following laboratory data:

RBC	2.30×10^{12}/L
Hb	7.8 g/dL (78 g/L)
Hct	0.24 L/L
MCV	104 fL
RDW	20
WBC	8.5×10^{9}/L
PLT	140×10^{9}/L

The differential was normal except for 2% metamyelocytes. Oval macrocytes, a few abnormal NRBC, and a few sideorocytes were seen. The bone marrow contained 3% blasts and exhibited hypercellularity with megaloblastoid development in erythroid cells.

8. What is the most probable MDS subgroup? (Objective 10)
   A. RCUD
   B. RARS
   C. RCMD
   D. RAEB
9. What other hematologic disorder does this peripheral blood picture resemble? (Objective 7)
   A. aplastic anemia
   B. megaloblastic anemia
   C. iron-deficiency anemia
   D. anemia of chronic disease
10. What laboratory test(s) would be helpful to distinguish MDS from megaloblastic anemia? (Objective 7)
    A. serum lysozyme
    B. serum ferritin
    C. serum folate and cobalamin
    D. combined esterase stain

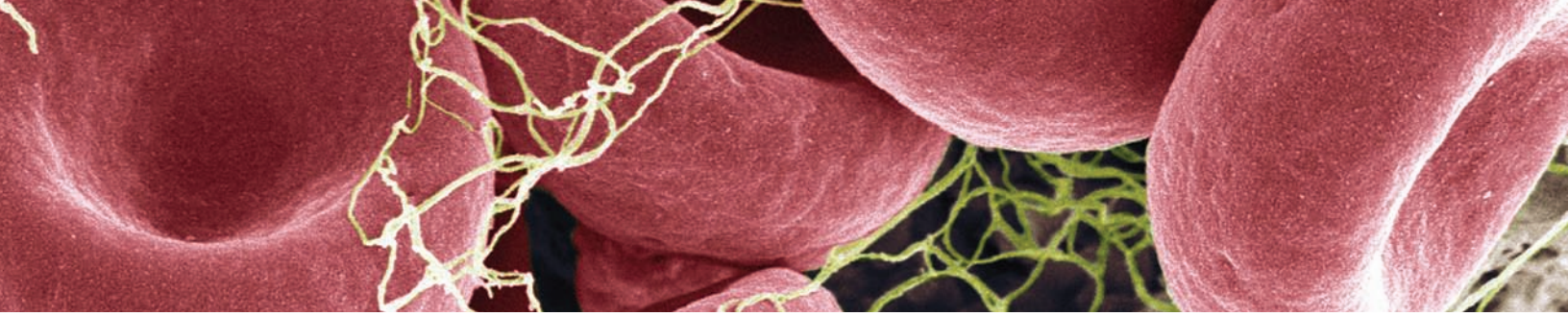
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Nimer SD. Myelodysplastic syndromes. *Blood*. 2008;111:4841–51.
2. Tormo M, Marugán I, Calabuig M. Myelodysplastic syndromes: an update on molecular pathology. *Clin Transl Oncol*. 2010;12:652–56.
3. Zhou J, Orazi A, Czader MB. Myelodysplastic syndromes. *Semin Diag Path*. 2011;28:258–72.
4. Davids MS, Steensma DP. The molecular pathogenesis of myelodysplastic syndromes. *Cancer Biol Ther*. 2010;10:309–19.
5. Bernasconi P. Molecular pathways in myelodysplastic syndromes and acute myeloid leukemia: relationships and distinctions—a review. *Br J Haematol*. 2008;142:695–708.
6. Kawankar N, Vundinti BR. Cytogenetic abnormalities in myelodysplastic syndrome: an overview. *Hematology*. 2011;16:131–38.
7. Nimer SD. An update on the molecular pathogenesis of myelodysplastic syndromes. *Biol Blood Marrow Transplant*. 2011;17(suppl 1):S11–4.
8. Nolte F, Hofmann WK. Myelodysplastic syndromes: molecular pathogenesis and genomic changes. *Ann Hematol*. 2008;87:777–95.
9. Odenike O, Anastasi J, Le Beau MM. Myelodysplastic syndromes. *Clin Lab Med*. 2011;31:763–84.
10. Haase D. Cytogenetic features in myelodysplastic syndromes. *Ann Hematol*. 2008;515–26.
11. Paulsson K, Johansson B. Trisomy 8 as the sole chromosomal aberration in acute myeloid leukemia and myelodysplastic syndromes. *Pathol Biol (Paris)*. 2007;55:37–48.
12. Caramazza D, Lasho TL, Finke CM et al. IDH mutations and trisomy 8 in myelodysplastic syndromes and acute myeloid leukemia. *Leukemia*. 2010;24(12):2120–22.
13. Qian Z, Joslin JM, Tennant TR et al. Cytogenetic and genetic pathways in therapy related acute myeloid leukemia. *Chem Biol Interact*. 2010;184: 50–7.
14. Aziz A, Baxter EJ, Edwards C et al. Cooperativity of imprinted genes inactivated by acquired chromosome 20q deletions. *J Clin Invest*. 2013;123:2169–82.
15. Shih AH, Levine RL. Molecular biology of myelodysplastic syndromes. *Semin Oncol*. 2011;38:613–20.
16. Tan PT, Wei AH. The epigenenomics revolution in myelodysplasia: a clinico-pathological perspective. *Pathology*. 2011;43:536–46.
17. Lin J, Yao DM, Qian J et al. Methylation status of fragile histidine triad *(FHIT)* gene and its clinical impact on prognosis of patients with myelodysplastic syndrome. *Leuk Res*. 2008;32(10):1541–45.
18. Issa JP. Epigenetic changes in the myelodysplastic syndrome. *Hematol Oncol Clin North Am*. 2010;24(2):317–30.
19. Liesveld JL, Lichtman MA. Myelodysplastic syndromes (clonal cytopenias and oligoblastic myelogenous leukemia). In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1249–76.
20. Bennett JM, Komrokji RS. The myelodysplastic syndromes: diagnosis, molecular biology and risk assessment. *Hematology*. 2005;10(suppl 1): 258–69.
21. Ma X, Does M, Raza A et al. Myelodysplastic syndromes: incidence and survival in the United States. *Cancer*. 2007;109:1536–42.
22. Scott BL, Deeg HJ. Myelodysplastic syndromes. *Annu Rev Med*. 2010;61:345–58.
23. Steensma DP, Bennett JM. The myelodysplastic syndromes: diagnosis and treatment. *Mayo Clin Proc*. 2006;81:104–30.
24. Jaffe ES, Harris NL, Stein H et al., eds. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. In: Kleihues P, Sobin LH, eds. *World Health Organization Classification of Tumours*. Lyon, France: IARC Press; 2001, 63–73.
25. Verburgh E, Achten R, Hagemeijer M et al. Additional prognostic value of bone marrow histology in patients subclassified according to the International Prognostic Scoring System for myelodysplastic syndromes. *J Clin Oncol*. 2003;15:273–82.
26. Della Porta MG, Malcovati L. Myelodysplastic syndromes with bone marrow fibrosis. *Haematologica*. 2011;96:180–83.
27. Mufti GJ, Bennett JM, Goasguen J et al. Diagnosis and classification of myelodysplastic syndrome: International Working Group on Morphology of myelodysplastic syndrome (IWGM-MDS) consensus proposals for the definition and enumeration of myeloblasts and ring sideroblasts. *Haematologica*. 2008;93:1712–17.
28. Epling-Burnette PK, List AF. Advancements in the molecular pathogenesis of myelodysplastic syndrome. *Curr Opin Hematol*. 2009;16:70–76.
29. Metzker ML. Sequencing technologies—the next generation. *Nat Rev Genet*. 2010;11:31–46.
30. Vardiman JW, Theile J, Arber DA et al. The 2008 World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–51.
31. Pagnucco G, Giambanco C, Gervasi F. The role of flow cytometric immunophenotyping in myelodysplastic syndromes. *Ann NY Acad Sci*. 2006;1089:383–94.
32. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood*. 2008;111:3941–67.
33. Ossenkoppele GJ, van de Loosdrecht AA, Schuurhuis GJ. Review of the relevance of aberrant antigen expression by flow cytometry in myeloid neoplasms. *Br J Haematol*. 2011;153:421–36.
34. Bennett JM, Catovsky D, Daniel MT et al. Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol*. 1982;51:189–99.
35. Harris NL, Jaffe ES, Diebold J et al. The World Health Organization classification of neoplastic diseases of the haematopoietic and lymphoid tissues: report of the Clinical Advisory Committee Meeting, Airlie House, Virginia, November 1997. *Histopatho*. 2000;36:69–87.
36. Hofmann WK, Koeffler HP. Myelodysplastic syndrome. *Annu Rev Med*. 2005;56:1–16.
37. Orazi A, Czader MB. Myelodysplastic syndromes. *Am J Clin Pathol*. 2009;132:290–305.
38. Tefferi A, Vardiman JW. Myelodysplastic syndromes: mechanisms of disease. *N Eng J Med*. 2009;361:1872–85.
39. Sill H, Olipitz W, Zebisch A et al. Therapy-related myeloid neoplasms: pathobiology and clinical characteristics. *Br J Pharmacol*. 2011;16:792–805.
40. Niemeyer CM, Kratz CP. Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukemia: molecular classification and treatment options. *Br J Haematol*. 2008;140:610–24.
41. Greenberg P, Cox C, LeBeau MM et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*. 1997;89:2079–88.
42. Greenberg PL, Sanz GF, Sanz MA. Prognostic scoring systems for risk assessment in myeloproliferative syndromes. *Forum*. 1999;9:17–31.
43. Cazzola M, Della Porta MG, Travagline E et al. Classification and prognostic evaluation of myelodysplastic syndromes. *Semin Oncol*. 2011;38:627–34.
44. Greenberg PL, Tuechler H, Schanz J et al. Revised international prognostic scoring system for myelodysplactic syndromes. *Blood*. 2012;120:245–65.
45. D'Alò F, Greco M, Criscuolo M et al. New treatments for myelodysplastic syndromes. *Mediterr J Hematol Infect Dis*. 2010;11(2):e2010–21.
46. Barzi A, Sekeres MA. Myelodysplastic syndromes: a practical approach to diagnosis and treatment. *Cleve Clin J Med*. 2010;77:37–44.
47. Garcia-Manero G. Myelodysplastic syndromes: 2011 update on diagnosis, risk-stratification, and management. *Am J of Hematol*. 2011;86:491–98.
48. Olnes MJ, Sloand EM. Targeting immune dysregulation in myelodysplastic syndromes. *JAMA*. 2011;23(305):814–19.
49. Blum W. How much? How frequent? How long? A clinical guide to new therapies in myelodysplastic syndromes. *Hematol Am Soc Hematol Educ Program*. 2010;314–21.
50. Al-Seraihy A, Ayas M, Al-Nounou R et al. Outcome of allogeneic stem cell transplantation with a conditioning regimen of busulfan, cyclophosphamide and low-dose etoposide for children with myelodysplastic syndrome. *Hematol Oncol Stem Cell Ther*. 2011;4:121–25.
51. Orazi A, Germing U. The myelodysplastic/myeloproliferative neoplasms: myeloproliferative diseases with dysplastic features. *Leukemia*. 2008;22:1308–19.
52. Hyjek E, Vardiman JW. Myelodysplastic/myeloproliferative neoplasms. *Semin Diagn Pathol*. 2011;28:283–97.
53. Chan RJ, Cooper T, Kratz CP et al. Juvenile myelomonocytic leukemia: a report from the 2nd International JMML Symposium. *Leuk Res*. 2009;33:355–62.



26 Acute Myeloid Leukemias

GAIL BENTLEY, MD
SUSAN J. LECLAIR, PHD

Objectives—Level I

By the end of this unit of study, the student should be able to:

1. Define *acute leukemia (AL)* and explain the difference between acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myeloproliferative neoplasm (MPNs), and myelodysplastic syndrome (MDS).
2. List and define the common variants of AML as defined by the World Health Organization (WHO) classification.
3. Describe and recognize the typical peripheral blood picture (erythrocytes, leukocytes, thrombocytes, and blasts) seen in AML.
4. Describe the bone marrow M:E ratio in AL.
5. Give the typical results of cytochemical stains in AML.
6. Identify Auer rods and describe their significance.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Compare and contrast the various presentations of AML.
2. Predict the most likely leukemia subtype based on patient history, physical assessment, and laboratory findings.
3. Correlate cellular presentation of AML with prognosis and common complications.
4. Correlate Wright stain morphology of the AML subgroups with cytochemical stains, flow cytometry, and genetic testing.
5. Evaluate peripheral blood results in relation to oncological therapy.
6. Evaluate patient data from the medical history and the laboratory results to determine whether a disorder can be classified, and, if not, specify the additional testing to be performed.
7. Explain the criteria used for the WHO classification of AML.

Chapter Outline

Key Terms

Acute leukemia (AL)
Auer rods
B lymphoid cell antigen
Dysplasia
Leukemia of ambiguous lineage
T lymphoid cell antigen
WHO classification

Background Basics

The information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review and have an understanding of the following concepts before starting this unit of study:

Level I

- Describe the origin and differentiation of hematopoietic cells. (Chapter 4)
- Summarize the maturation, differentiation, and function of leukocytes. (Chapter 7)
- Outline the classification and general laboratory findings of acute leukemias. (Chapter 23)
- List and describe the criteria used to differentiate acute leukemias from other hematologic neoplasms. (Chapter 23)

Level II

- Summarize the role of oncogenes and growth factors in cell proliferation, differentiation, and maturation. (Chapters 2, 4, and 23)
- Describe the role of molecular analysis in diagnosing and treating acute leukemia. (Chapters 23, 42)
- Describe the use of immunophenotyping in acute leukemia. (Chapters 23, 40)
- Describe the role of cytogenetics in diagnosis, treatment, and prognosis of acute leukemia. (Chapters 23, 41)

CASE STUDY

We will refer to this case study throughout the chapter.

Guillermo, a 34-year-old Latin-American male, had been in excellent health until he was seen at a neighborhood clinic and was prescribed penicillin for a mild sore throat. He was able to return to work and felt better. After several days he developed a fever and experienced easy bruising. He noticed other bleeding symptoms including gingival bleeding and petechiae. He reported to the emergency room. A CBC revealed a WBC count of 26.2×10^9/L and 6% blasts. He was admitted to the hospital for further evaluation.

Consider what additional laboratory testing could assist in diagnosing Guillermo.

OVERVIEW

This chapter describes the acute myeloid (also referred to as *myelogenous*) leukemias (AMLs). It begins with the general laboratory findings in AML followed by a specific description of each subgroup in the World Health Organization (WHO) classification. Clinical and laboratory findings are described with an emphasis on defining characteristics. The chapter concludes with the current types of therapy used to treat AML.

INTRODUCTION

All **acute leukemias (ALs)** are stem cell disorders characterized by malignant neoplastic proliferation and accumulation of immature and nonfunctional hematopoietic cells in the bone marrow. The neoplastic cells show increased proliferation and/or decreased programmed cell death (apoptosis). The net effect is expansion of the leukemic clone and a decrease in normal cells.[1]

Acute leukemia is a clonal expansion of a single transformed cell; therefore, all ALs begin long before any clinical signs and symptoms appear. A tumor burden of 10^{12} cells is believed to be sufficient for recognizable signs and symptoms. Lethal levels of tumor burden occur at neoplastic cell numbers of $10^{13–14}$ or higher. As the tumor burden expands, the normal functional marrow cells decrease. The classic triad of anemia, infection, and bleeding seen in acute leukemia occur as a result of "normal" hematopoietic cell cytopenias. Death often occurs from either infection or hemorrhage in weeks to months unless therapeutic intervention occurs.[2]

The two major categories of acute leukemias are classified according to the origin of the cell with the primary defect: AML and acute lymphoblastic leukemia (ALL). Acute lymphoblastic leukemia is also referred to as *lymphoblastic leukemia* (omitting *acute* because this is considered redundant). If the defect primarily affects the maturation and differentiation of the common myeloid progenitor (CMP) cell, the leukemia is classified as AML. If the defect primarily affects the common lymphoid progenitor (CLP) cell, the leukemia is classified as ALL.

The acute leukemias are classified into subtypes. The most reliable parameters for defining and classifying neoplastic cells in AML

into subtypes are cell markers (cell surface or internal antigens) defined by immunologic probes and genetic abnormalities identified with cytogenetic and molecular studies (Chapters 40–42). In the late 1970s, the first internationally accepted classification for the acute leukemias, the French-American-British (FAB) classification, was based on a combination of neoplastic cell morphology and cytochemical cellular reactions.[3] This classification remained essentially unchanged until 1999 when the WHO and the International Society of Hematology proposed a new classification.[4] Health care practitioners have dropped the use of the old FAB system in favor of the **WHO classification**.

ETIOLOGY AND PATHOPHYSIOLOGY

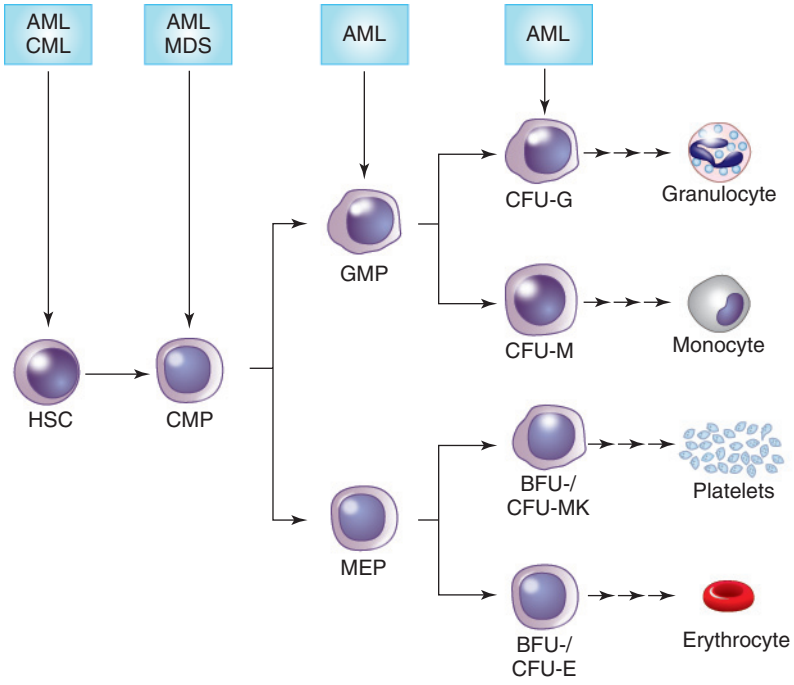
AML is a disease characterized by two fundamental cellular features: the ability to proliferate continuously and aberrant or arrested development[1] (Figure 26-1 ■). Excessive proliferation can be the result of mutations affecting growth factors, growth factor receptors, signaling pathway components, and transcription factors that regulate genes involved in cell survival and proliferation (Chapter 23). The majority of the genetic mutations that have been identified in AML involve transcription factors and other signaling pathway molecules. More than half of the cases of AML display cytogenetic abnormalities. Most are balanced, reciprocal chromosomal translocations with many of the translocation break points located at the loci for genes encoding transcription factors. The most common consequence of the translocation is the generation of a fusion gene that codes for a novel fusion protein. The fusion proteins that are formed usually alter the normal function of one or both of the rearranged genes and modify the normal programs of cell proliferation, differentiation, and survival. In addition, other types of genetic abnormalities (e.g., epigenetic alterations) likely interact with the cytogenetic mutations (called the *multistep origin* of malignancy), resulting in the full leukemic transformation.[1]

LABORATORY FINDINGS

Peripheral Blood

The peripheral blood picture is quite variable in AML. Although it is traditional to describe leukemias as having elevated leukocyte counts, 50% of the cases have decreased counts or counts within the reference interval at the time of diagnosis. The leukocyte count ranges from $<1 \times 10^9$/L to $>100 \times 10^9$/L. Regardless of the leukocyte concentration, the presence of blasts in the blood smear suggests the AL diagnosis. The current WHO definition of AL requires ≥20% blasts in the peripheral blood or bone marrow.[5] Cases must be classified based on a combination of immunologic, cytogenetic and molecular genetic methods, and cytochemical tests (Chapter 23). Typically, the myeloblast seen in AML is approximately 20 mcM (μm) in diameter with variably prominent nucleoli in a nucleus composed primarily of dispersed chromatin (euchromatin or transcriptionally active DNA). A Golgi apparatus is present but is not easily visualized. Although blasts normally do not have granules visible by bright-field microscopy, neoplastic blasts do not always develop normally and can have granules in the RNA-rich cytoplasm (Chapter 25).

Erythrocytes are typically decreased, and a hemoglobin value <10 g/dL is common. Erythrocytes can be slightly macrocytic



■ **FIGURE 26-1** AML may originate from any of the cells that fall within the paths of the downward arrows. Importantly, the AML cell of origin acquires the capacity for self-renewal and maturation arrest.

AML = acute myelogenous leukemia; BFU = burst-forming unit; CFU = colony-forming unit; CML = chronic myelogenous leukemia; CMP = common myeloid progenitor cell; E = erythroid; G = granulocytic; GMP = granulocyte monocyte progenitor; HSC = hematopoietic stem cell; MDS = myelodysplastic syndrome; MEP = megakaryocyte erythrocyte progenitor; M = monocytic; MK = megakaryocytic

because of their inability to successfully compete with neoplastic cells for folate or cobalamin and/or early release of marrow reticulocytes. The red cell distribution width (RDW) often is elevated. Erythrocyte inclusions such as Howell-Jolly bodies, Pappenheimer bodies, and basophilic stippling, reflective of erythrocyte maturation defects, can be present. Nucleated erythrocytes can be present in proportion to the anemia or marrow damage.

Platelets are typically decreased. Hypogranular platelets and occasional enlarged forms (giant platelets) can be present. As the disease progresses, more immature platelet forms, such as megakaryocytic fragments, can be seen. The platelet count might not correlate with the potential complication of bleeding because qualitative platelet defects also may be present.

If the physician suspects AL but no blast cells are detected in the peripheral blood smear or if the leukocyte count is low ($<2 \times 10^9$/L) a buffy-coat smear may be prepared and often reveals the presence of blast cells when they are present in very low concentrations. Finding blasts with azurophilic granules is helpful in identifying the myeloid nature of the leukemia. The presence of **Auer rods** (fused or coalesced primary granules) in blasts excludes a diagnosis of ALL. Auer rods are primarily found in myeloblasts and on rare occasions in monoblasts or more differentiated monocytic or myelocytic cells (promyelocytes). They are not found in lymphoid blasts.

Other abnormal findings on the blood smear can include monocytosis and neutropenia. Monocytosis frequently precedes overt leukemia. Neutrophils can demonstrate signs of **dysplasia** including hyposegmentation, hypogranulation, and small nuclei with hypercondensed chromatin. Signs of myelodysplasia are especially common in promyelocytic leukemia.[6] Eosinophils and basophils can be mildly to markedly increased. When present, basophilia can help to differentiate leukemia from a leukemoid reaction. Absolute basophilia is not present in a leukemoid reaction. A blast phase of chronic myeloid leukemia (CML) should be excluded by evaluating *BCR/ABL1*.

Bone Marrow

Bone marrow (BM) testing should include both aspirate and biopsy specimens. The quality of marrow specimen is critical for all subsequent analyses. Typically, the BM presentation is hypercellular with decreased fat content (relative to age-related normals), a predominance of blasts, and sometimes an increase in fibrosis. According to the WHO criteria for AL, blasts must compose ≥20% of the nonerythroid nucleated cells to distinguish AL from myelodysplastic syndromes. Frequently, the blast count is close to 100%. Auer rods are present in BM blasts in about half of AML cases.

Cells can be clumped together, occasionally forming sheets of infiltrate that disturb the usual marrow architecture. In addition to light microscopic morphologic evaluation, BM samples should be sent for flow cytometry, cytogenetics, and molecular genetics.

☑ CHECKPOINT 26-1

What results would you expect to find on the CBC and differential in a suspected case of AL?

Other Laboratory Findings

Other laboratory findings can reflect the increased proliferation and turnover of cells. Hyperuricemia and increased lactate dehydrogenase (LD) are common findings resulting from the increased cell turnover. When present, hypercalcemia is thought to be caused by increased bone resorption associated with leukemic proliferation in the bone marrow. Increased serum and urine muramidase are typical findings in leukemias with a monocytic component.

CASE STUDY (continued from page 501)

Admission laboratory data on Guillermo are as follows:

RBC	3.2×10^{12}/L	**WBC Differential**	
Hb	9.7 g/dL	Blasts	6%
Hct	30.5 L/L (30%)	Promyelocytes	79%
PLT	31×10^9/L	Myelocytes	5%
WBC	26.2×10^9/L	Lymphocytes	11%

Erythrocyte morphology: Erythrocytes are normochromic and normocytic with rare schistocytes seen.

1. What clues do you have that this patient could have an acute leukemia?
2. Based on the presenting data, what additional testing might be of value?

CLASSIFICATION

The 2008 WHO classification is the result of a worldwide consensus on classification of the hematopoietic neoplasms and expands the parameters used to classify the neoplastic disorders.[7–10] The parameters include not only microscopic morphology and cytochemistry but also flow cytometry, cytogenetics, molecular genetic abnormalities, and clinical findings. In this classification, a finding of ≥20% blasts establishes the diagnosis of AL. The WHO major AML subgroups, each with variants or subtypes, are:

- *AML with recurrent genetic abnormalities* is further differentiated into subgroups.
- *Therapy-related myeloid neoplasms* are AMLs distinctly different from de novo (primary) AML in terms of response to therapy and prognosis.
- *AML with myelodysplasia-related changes* are variants with and without prior history of myelodysplastic syndrome (MDS).
- *AML not otherwise specified* includes any AML not included in the preceding subgroups.
- *Myeloid sarcoma*
- *Myeloid proliferations related to Down syndrome*
- *Blastic plasmacytoid dendritic cell neoplasm*

For the complete WHO AML classification with subgroups, see Table 26-1 ★.

★ **TABLE 26-1** 2008 WHO Classification of Acute Myeloid Leukemias (AML)

Group	Subgroups
1. AML with recurrent genetic abnormalities	
AML with balanced translocations/inversions	AML with t(8;21)(q22;q22.3), *RUNX1/RUNX1T1*
	AML with inv(16)(p13q22) or t(16;16)(p13;q22), CBFβ-MYH1
	AML with t(9;11)(p22;q23); MLLT3-MLL
	APL (promyelocytic) with t(15;17)(q22;q12), PML/RARα, and AML with a variant RARα translocation
	AML with t(6;9) (p23;q34); DEK-NUP214
	AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
	AML with t(1;22)(p13;q13); RBM15-MKL1
AML with gene mutations	AML with mutated NPM1
	AML with mutated CEBPA
2. AML with myelodysplasia-related changes	AML arising from a previous myelodysplastic or myelodysplastic/myeloproliferative neoplasm
	AML with an MDS-related cytogenetic abnormality
	AML with multilineage dysplasia
3. Therapy-related myeloid neoplasms Includes (t-AML), (t-MDS), and (t-MDS/MPN)	Alkylating agent related
	Ionizing radiation therapy related
	Topoisomerase II inhibitors
	Others
4. Myeloid sarcoma	Tumor mass consisting of myeloid blasts with or without maturation occurring in an extramedullary site
5. Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis
	Myeloid leukemia associated with Down syndrome
6. Blastic plasmacytoid dendritic cell neoplasm	Derived from precursors of plasmacytoid dendritic cells
7. AML not otherwise specified	AML minimally differentiated
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic and monocytic leukemia
	Acute erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis

Identification of Cell Lineage

Because blast cells are immature cells, they are often difficult to identify by morphology alone using light microscopy. Cytochemistry, molecular testing, and immunophenotyping give additional information that can help define cell lineage (Chapters 40, 41, 42). When both cytochemistry and immunophenotyping are used, most AL cases can be classified as being of lymphoid or myeloid origin. Rarely, a population of malignant blasts is cytochemically negative by conventional methods and nonreactive with both lymphoid and myeloid monoclonal antibodies. Such leukemias are classified as undifferentiated.[5]

The acute **leukemias of ambiguous lineage** encompass leukemias that show no clear evidence of differentiation to a single lineage. This group includes leukemias without lineage-specific antigens (acute undifferentiated leukemia) and leukemias that express antigens of more than one lineage, making it impossible to determine any one lineage (mixed phenotype acute leukemias, MPAL). These leukemias are discussed in Chapter 27.

Cytochemistry

The common cytochemical stains include the myeloperoxidase (MPO), Sudan black B (SBB), naphthol AS-D chloroacetate esterase (specific esterase), and α-naphthyl esterases (nonspecific esterase) (Chapter 34). These stains are still the foundation of cytochemical analysis. Granulocytic cells stain positive with MPO and with SBB; lymphoblasts are negative. Thus, these stains help differentiate the acute myeloid leukemias from the acute lymphoblastic leukemias. The esterase stains help differentiate precursor granulocytic cells from precursor monocytic cells. Granulocytic cells stain positive with naphthol AS-D chloroacetate esterase, and cells of monocytic lineage stain positive with nonspecific esterase. Most institutions have begun using immunophenotyping and cytogenetics as first-line tests to define cell lineage. However, cytochemistry can be helpful in cases not defined by these methods and in identifying some subgroups.[11]

Flow cytometry or immunohistochemistry can be used to demonstrate terminal deoxynucleotidyl transferase (TdT) in individual cells. Although originally thought to be a lymphoid specific marker,

TdT is found on more immature hematopoietic cells, sometimes including those of myeloid lineage.[12] Therefore, TdT cannot be the sole determinant of lymphoid lineage.

CASE STUDY *(continued from page 503)*

3. Based on the peripheral blood examination, what cytochemical stain results would you expect to find on Guillermo's neoplastic cells?

Immunophenotyping

Immunophenotyping by flow cytometry has become a necessary component of AL classification, particularly when the morphological appearance and cytochemical reactions do not clearly define cell lineage or when the presence of more than one neoplastic cell population is suspected.[13] Immunophenotyping by flow cytometry follows a specific sequence of testing with monoclonal antibodies.[14] The use of extensive panels is costly and time consuming. In most cases, lineage can be determined using a limited, representative panel of monoclonal antibodies. Common cell markers used in identification include CD2, CD3, CD4, CD5, CD7, CD10, CD13, CD14, CD15, CD16, CD19, CD22, CD33, MPO, HLA-DR, CD34, CD45, CD56, CD64, and CD117.[15]

The first panel of monoclonal antibodies should differentiate AML from ALL and T-cell ALL (T-ALL) from B-cell ALL (B-ALL). Use of a panel of antibodies such as those listed in Table 26-2 ★ can usually discriminate AML from ALL. Individual facilities have their own preferred panels of antibodies. Panels should include typing for the myeloid antigens, the **B lymphoid cell antigens** (CD19, CD20, CD22, and CD79a), and the **T lymphoid cell antigens** (CD2, CD3, CD5, and CD7). Human leukocyte antigen (HLA-DR) is present on both myeloid and B lymphoid cells. Several aberrant antigens are sometimes found on neoplastic cells (e.g., the CD7 [T-lymphoid] antigen can be found on neoplastic myeloid cells in AML). The CD34 marker is also present on the least differentiated myeloid cells and early lymphoid cells. The monoclonal antibodies that react with most cases of AML include CD13, CD15, CD33, CD64, and CD117.[15] In addition, the monoclonal antibody that identifies MPO is helpful, especially when cytochemistry for MPO is negative.

★ **TABLE 26-2** Differentiation of ALL from AML Using Immunophenotyping with Selected Monoclonal Antibodies

	Cell Marker				
Leukemia Type	HLA-DR	CD13, CD33	CD19, CD20, CD22, CD79a	CD10	CD2, CD3, CD5, CD7
AML	+	+	−	−	−
B lymphocyte	+	−	+	+	−
T lymphocyte	−	−	−	±	+

The CMP cell is capable of differentiation into granulocytes, erythrocytes, monocytes, and megakaryocytes. Thus, if the neoplastic clone has "early" myeloid antigens, a second panel of monoclonal antibodies should include antibodies to subtype the AML into granulocytic, monocytic, erythrocytic, and megakaryocytic lineages (Table 26-3 ★).

CASE STUDY

4. If the cells from Guillermo's BM were immunophenotyped, which of these—CD13, CD33, CD34, CD2, CD7, CD10, CD19—would you expect to be positive?

Cytogenetic Analyses

Two-thirds of patients with AML have detectable cytogenetic abnormalities that include aneuploidies (variation in total chromosome number) and translocations.[1] Commonly observed aneuploidies include trisomy 8, monosomy 7, monosomy 21, trisomy 21, and loss of an X or Y chromosome. Translocations result in fusion genes that are either beneficial for proliferation and survival or disrupt differentiation and maturation. Additional genetic abnormalities can develop in subclones as the disease progresses.

Characteristic, nonrandom cytogenetic abnormalities are observed in the WHO classifications of AML with recurrent genetic abnormalities and frequently in therapy-related myeloid neoplasms. These nonrandom chromosome abnormalities are discussed in subsequent sections. If the expected abnormal karyotype is not found, fluorescence in situ hybridization (FISH) or molecular analysis is sometimes helpful.

☑ CHECKPOINT 26-2

Explain why molecular analysis is not performed on all suspected cases of acute leukemia.

Assessment of Bone Marrow

When a diagnosis of AML, MDS, or MPN is suspected, the first step is to estimate the BM cellularity followed by assessing the percentage of blasts. Further evaluation of the BM depends on the number of erythroblasts present (>50% or <50% of all nucleated marrow cells). If erythroblasts compose ≥50% of all nucleated BM cells, the percentage of nonerythroid blast cells (i.e., myeloblasts, monoblasts) is determined by performing a differential count excluding the erythroid cells. If ≥20% myeloblasts are in the nonerythroid population of cells, the AML subcategory is defined as acute erythroid leukemia (erythroid/myeloid variant). If <20% blasts are in the nonerythroid population, the diagnosis may be the acute erythroid leukemia (pure erythroid leukemia variant). If <50% erythroblasts are in the bone

★ TABLE 26-3 Morphologic, Immunophenotypic, and Cytochemical Results Used to Classify AML Subcategories

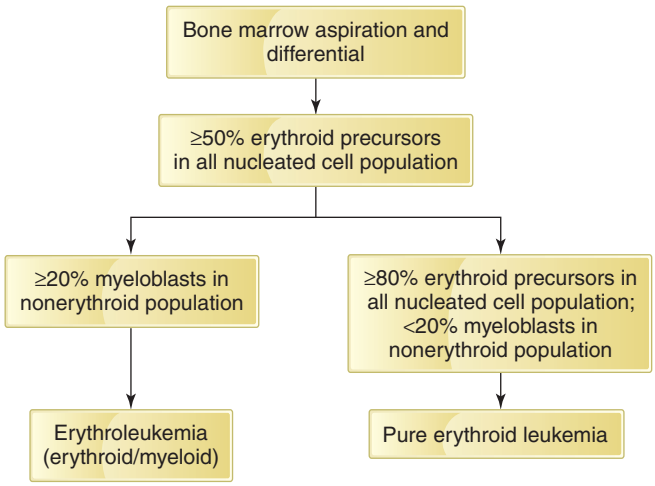
			Cell Markers with Monoclonal Antibodies									
AML Subgroup	**Morphology**	**Cytochemistry**	**HLA-DR**	**CD117**	**CD34**	**CD13**	**CD33**	**CD11b**	**CD14**	**CD71 Glycophorin A**	**CD41, CD42, CD61**	**Other Markers That May Be Present**
AML not otherwise specified												
AML minimal differentiated	Minimal evidence of myeloid differentiation No maturation seen Agranular cytoplasm	Myeloperoxidase− (<3% of blasts) Sudan black B−(<3% of blasts) Specific esterase−	+	+++	++	+	+	−	−	−	−	CD7, CD38 TdT[a]
AML without maturation	Evidence of myeloid differentiation, no maturation seen, Auer rods or some granulation	Myeloperoxidase+ (>3% of blasts) Sudan black B+ (>3% of blasts) Specific esterase+	+	+++	+	++	+	+/−	+	−	−	CD7, Lysosym
AML with maturation	All stages of neutrophil maturation Pseudo–Pelger-Huët Hypogranulation Esoinophilic precursors possible	Myeloperoxidsae+ Sudan black B+ Specific esterase+	+/−	++	+/−	++	+++	−	−	−	−	CD4, CD15, CD19, Lysosyme
Acute myelo-monocytic leukemia (AMML)	Neutrophil and monocytic precursors present Vacuolization	Myeloperoxidase+ Nonspecific esterase+/− Specific esterase+	−	+	−	++	+++	+	+/−	−	−	CD4, CD11c CD36, CD64, CD68, Lysosyme
Acute monoblastic /monocytic leukemia (AMoL)	Monoblast/monocyte dominance Hemophagocytosis present Nuclear lobulation	Myeloperoxidase− Nonspecific esterase+/− Specific esterase+	+	+	−	+	+++	+	+	−	+/−	CD11c, CD15 CD65,CD4 CD64, Lysosyme
Acute erythroid leukemia (AEL)	> 50% erythroid precursors and > 20% myeloblasts in the nonerythroid cell population Megaloblastoid morphology Auer rods+/−	Myeloperoxidase+ Sudan black B+ Nonspecific esterase+/− PAS+(erythroblasts)	+/−	+	−	+	+++	−	−	+	−	−
Acute megakaryo-blastic leukemia (AMkL)	Cytopenia with or without thrombocytopenia Basophilic agranular blasts with pseudopods Micro-megakaryocytes	Myeloperoxidase− Sudan black B− Nonspecific esterase+/− Specific esterase− PAS+ Platelet peroxidase+	+	+	+	−	+	−	−	−	+/−	−

AML Subgroup	Morphology	Cytochemistry	Cell Markers with Monoclonal Antibodies									
			HLA-DR	CD117	CD34	CD13	CD33	CD11b	CD14	CD71 Glycophorin A	CD41, CD42, CD61	Other Markers That May Be Present
Acute basophilic leukemia (ABL)	Basophilic precursors Vacuolization	Metachromatic positivity with toluidine blue	+	+	+	−	+	−	−	−	++	CD123, CD203, CD9
Acute panmyelosis with myelofibrosis	Pancytopenia Dysplastic changes in neutrophils and platelets	Myeloperoxidase+	+	+	+	+	+	−	−	+/−	+/−	Lysozyme
Therapy related myeloid neoplasms (t-MDS, t-AML, t-MDS/MPN)												
	Most have multilineage dysplasia Basophilia is often present Pancytopenia or isolated cytopenia Associated with unbalanced loss of genetic material often involving chromosomes 5 and or 7	Myeloperoxidase+ Sudan black B+ Increased iron in ring formation PAS+ Specific esterase+ Nonspecific esterease+	No consistent phenotype									CD13, CD33, CD34, CD7, CD56
AML with myelodysplasia-related changes												
Following MDS or MDS/MPNs With a cytogenetic abnormality characteristic of MDS With multilineage dysplasia	Dysplasia required in ≥50% of two cell lines Hypogranular neutrophils, pseudo-Pelger-Huët nuclei Dyserythropoiesis with megaloblastoid changes, ringed sideroblasts, nuclear fragments or vacuoles Dysmegakaryopoiesis with micromegakaryocytes, hypolobulated nuclei, and other dysplastic signs	Myeloperoxidase+ Sudan black B+ Increased iron in ring formation PAS+	No consistent phenotype									CD13, CD33, CD34, CD7, CD56, Multi-drug resistance glycoprotein receptor (MDR-1)

[a]TdT is usually used to identify early lymphoid precursors but can also be found in 10–20% of AML

Specific esterase = Napthol AS-d chloroacetate esterase; nonspecifi esterase = Alpha-naphthyl esterase

marrow, it is not necessary to exclude the erythroid cells from the differential count in determining the percentage of myeloblasts or monoblasts (Figure 26-2 ■). The diagnosis of myeloid AL is made when ≥20% of all nucleated BM cells are myeloid blasts. Additional assessment of the subtypes of AML evaluates only the myeloid cells, not lymphocytes, plasma cells, mast cells, macrophages, or nucleated erythrocytes.



■ **FIGURE 26-2** Suggested steps in the analysis of a bone marrow aspirate to reach a diagnosis. If there are <50% erythroblasts and >20% of all nucleated cells are leukocytic blasts, the diagnosis is AML. If there are <50% erythroblasts and <20% of all nucleated cells are leukocytic blasts, the diagnosis may be MDS or MPN.

WHO Classification of AML

AML with Recurrent Genetic Abnormalities

The AML with recurrent genetic abnormalities classification is used when chromosome abnormalities, usually balanced (reciprocal) translocations are found. In most cases, the chromosomal rearrangements create a fusion gene, encoding a novel fusion (chimeric) protein. Molecular techniques (RT-PCR) have a higher sensitivity than cytogenetics for diagnosing and following the progression of the disease. The AMLs in this category generally have a high rate of complete remission and a more favorable prognosis.

AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*

This subcategory is found in 5–10% of AML cases (previously classified as *AML with maturation*). It occurs predominantly in children and adults younger than 60 years of age.[16] Myeloblasts are usually large with abundant basophilic cytoplasm containing numerous azurophilic granules, perinuclear clearing, and Auer rods. Some blasts may show very large granules (pseudo-Chédiak-Higashi granules). Promyelocytes, myelocytes, and mature neutrophils with variable dysplasia are usually present in the bone marrow. Dysplasia in other cell lines is uncommon. Eosinophilia can be present (Table 26-4). Cytochemical

★ **TABLE 26-4** Cytogenetic and Morphologic Results Used to Classify AML with Recurrent Cytogenetic Abnormalities

AML with recurrent genetic abnormalities	Morphology
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*	Maturation in the neutrophil lineage
	Auer rods, pseudo–Pelger-Huët nuclei
AML with inv(16)(p13q22) or t(16;16)(p13;q22) or t(16;16)(p13.1;q22); *CBFβ-MYH11*	Maturation in both the neutrophil and monocytic lineage, abnormal eosinophils (AMML Eo)
	Eosinophils ≥5%
	Abnormal eosinophilic granules present in promyelocyte or myelocyte stage
AML with t(9;11)(p22;q23); *MLLT3-MLL*	Monoblasts and promonocytes predominate, can see vacuoles and granules
Acute promyelocytic leukemia with t(15;17)(q22;q12), *PML-RARA* and variant translocations	Hypergranular variant
	Promyelocytes dominate
	Multiple Auer rods in one cell
	Microgranular variant
	bi-lobed nuclei
	No visible granularity
AML with t(6;9)(p23;q34); *DEK-NUP214*	With or without monocytic features and often associated with basophilia and multilineage dysplasia
AML with inv(3)(q21;q26) or t(3;3) (q21;q26.2); *RPN1-EVI1*	Associated with elevated platelets and atypical megakaryocytes (mono or bilobed) and multilineage dysplasia
AML with t(1;22)(p13;q13), *RBM15-MLK1*	Megakaryocytic lineage with maturation
AML with Mutated *NPM1*	Myelomonocytic or monocytic features presents in older people with a normal karyotype
AML with mutated *CEBPA*	Myeloid leukemia with or without maturation.
	Can show myelomonocytic or monoblastic features

and immunophenotypic results are typical for myeloblasts (Appendix B). The leukemic "cell of origin" is believed to be the hematopoietic stem cell (HSC).

The t(8;21)(q22;q22) translocation repositions the 5′ region of the *RUNX1* gene (chromosome 21, previously termed *AML1*) with the 3′ region of the *RUNX1T1* gene (chromosome 8; also called *ETO*). The result is a fusion protein called *RUNX1-RUNX1T1* (also known as *RUNX1-ETO*). *RUNX1* is a member of a family of genes called *core-binding factor-α* (*CBFα*). CBFα proteins such as RUNX1 normally form a heterodimeric complex with CBFβ proteins in order to bind to DNA as functional transcription factors. The RUNX1-RUNX1T1 fusion protein acts as a transcriptional repressor by blocking the normal DNA binding function of RUNX1, resulting in its inability to regulate cellular differentiation. Furthermore, the RUNX1-RUNX1T1 fusion leads to the activation of other genes involved in cellular proliferation. The outcome is blocked differentiation[17] and increased proliferation of the leukemic cells.[18]

AML with inv(16)(p13;q22) or t(16;16)(p13;q22); *CBFß-MYH11*

This subcategory is identified in 7% of patients with AML[19] and generally presents with monocytic and granulocytic maturation and the presence of abnormal eosinophils in the bone marrow. The most striking abnormality of the eosinophils is the presence of immature (basophilic) granules that would normally predominate at the promyelocyte and myelocyte stages (Chapter 7). The immunophenotypic and cytochemical results reflect the presence of both neutrophilic and monocytic lineages (Appendix B). The leukemic cell of origin is believed to be an HSC.

The inv(16)(p13;q22) and t(16;16)(p13;q22) both result in the fusion of the core-binding factor gene *CBFβ* (16q22) to the smooth muscle myosin heavy chain gene *SMMHC* (*MYH11*) at 16p13.1. Because of difficulties in correctly identifying these mutations with traditional cytogenetics, FISH or RT-PCR may be necessary to document the mutation. The CBFβ-MYH11 fusion protein binds to RUNX1 and represses its function as a transcription factor.[20]

AML with t(9;11)(p22;q23); *MLLT3-MLL*

These leukemias are usually associated with monocytic features (monoblasts and promonocytes). They can occur at any age but are more common in children (up to 25% of childhood AML cases and 2–5% of all AML cases).[21] Identical cytogenetic abnormalities can also be found in therapy-related AML (see section "Therapy-Related Myeloid Neoplasms"). Patients can have extramedullary myeloid (monocytic) sarcomas (extramedullary leukemia) and tissue infiltration (gingiva, skin) and may present with disseminated intravascular coagulation. Monoblasts and promonocytes can have scattered azurophilic granules and vacuoles and give cytochemical and phenotypic results typical for the monocytic lineage (Table 26-4). The leukemic cell of origin is believed to be the HSC.

The *MLL* gene (11q23) is involved in a number of leukemia-associated translocations with different partner chromosomes. The MLL protein is a DNA-binding protein that interacts with other nuclear proteins and permits the association of transcription factors that help regulate transcription. The most common translocations involving 11q23 seen in childhood AML are t(9;11)(p21;q23), t(11;19)(q23;p13.1), and t(11;19)(q23;p13.3). More than 80 different translocations involving *MLL* are described with >50 different translocation partner genes. Up to one-third of *MLL* translocations in AML are not detectable by conventional cytogenetics, so FISH or molecular studies must be performed.[10]

☑ CHECKPOINT 26-3

What does *recurrent genetic abnormality* mean in the context of AML, and what is the general outcome of these abnormalities?

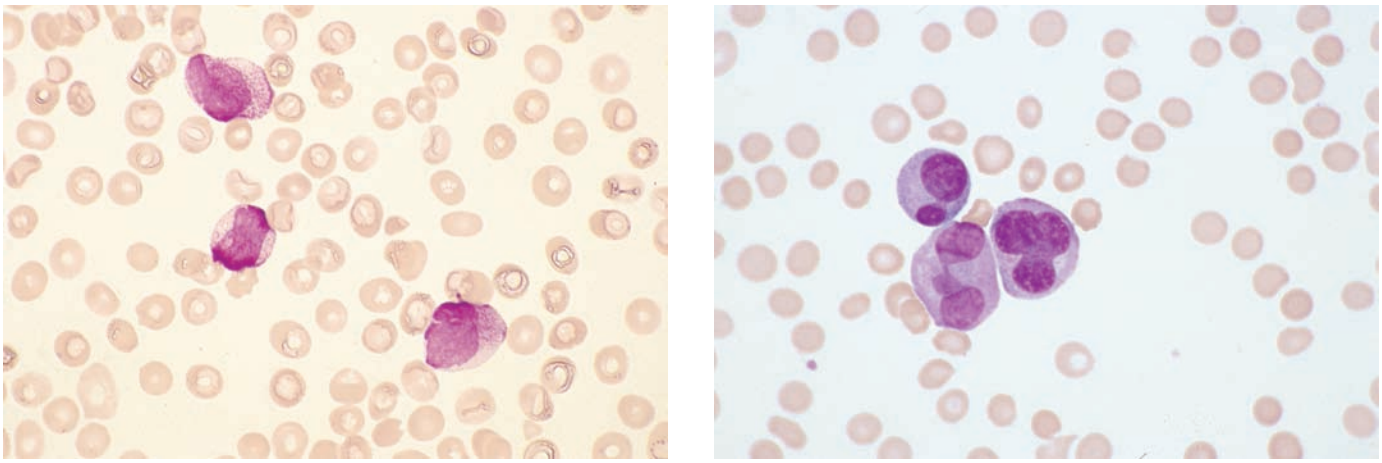
AML with t(15;17)(q22;q12); (PML/RARα) and Variants

Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12) is an AML in which abnormal promyelocytes predominate. Both hypergranular ("typical" APL) and hypogranular or microgranular presentations can be seen. APL can occur at any age, but most patients are adults in middle life. APL constitutes 5–8% of AML.[10]

The presenting signs for both hypergranular and hypogranular APL often include acute disseminated intravascular coagulation (DIC).[22] See Chapter 34 for the pathophysiology of DIC. The most common clinical finding at initial diagnosis is bleeding. The release of procoagulant material from promyelocytic granules likely initiates DIC, a serious complicating factor of the disease.[23] Cytotoxic therapy for APL can potentiate or aggravate this complication. Not only do the lysed promyelocytes release large amounts of procoagulant material but also tissue factor–containing particles are released from dying cells during cytotoxic therapy. Evidence of secondary fibrinolysis as a component of the DIC syndrome is also present. Heparin therapy can be administered with initiation of chemotherapy to prevent or modulate the DIC. Other abnormalities of coagulation can be present.

Hypergranular APL. Most patients with the hypergranular subtype are leukopenic or exhibit only slightly increased leukocyte counts. Most cells in the BM are abnormal promyelocytes with heavy azurophilic granulation. The granules can be so densely packed that they obscure the nucleus (Figure 26-3a ■). Some cells filled with fine dustlike granules also can be present. Cells with multiple Auer rods, sometimes occurring in bundles, are characteristic with cytoplasm that is frequently clear and pale blue, but cells can contain only a few azurophilic granules or lakes of clear pink material[24] (Figure 26-4 ■). In some cases, the typical hypergranularity of promyelocytes is less evident in the peripheral blood than in the bone marrow.[25] The nucleus varies in shape but is often folded or indented or sometimes bilobed. Often a large number of promyelocytes appear to be disrupted on the blood smear with free azurophilic granules and Auer rods intermingled with intact cells. Anemia and thrombocytopenia are typical findings.

Microgranular APL Variant. In contrast to typical APL, the leukocyte count in microgranular APL is usually markedly increased.[26] The predominant cell in the peripheral blood is a promyelocyte with a bilobular, reniform, or multilobed nucleus (resembling that of a monocyte)

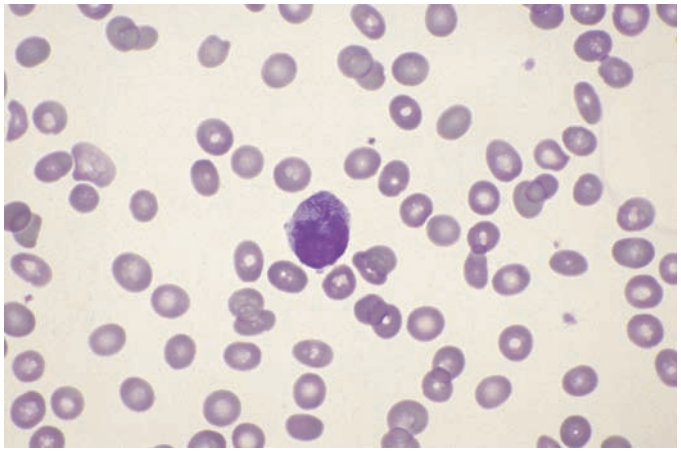


a **b**

■ **FIGURE 26-3** **(a)** Peripheral blood film from a patient with acute promyelocytic leukemia (APL), hypergranular variant. These promyelocytes have an irregularly shaped nucleus and numerous azurophilic granules. **(b)** Peripheral blood film from a patient with APL, microgranular variant. Note the bilobed nuclei and absent or fine granules in these abnormal promyelocytes (Wright-Giemsa stain, 1000× magnification).

and cytoplasm with an apparent paucity of granules on Wright-stained smears (Figure 26-3b). The apparent absence of granules is because of their submicroscopic size, but they are readily visible with cytochemistry (myeloperoxidase, MPO positive). The nuclear chromatin is fine with nucleoli often visible. A small abnormal promyelocyte with a bilobed nucleus, deeply basophilic cytoplasm, and sometimes cytoplasmic projections is present as a minor population of cells in most cases and occasionally is the predominant cell. When cytoplasmic projections are present, the cells can resemble megakaryoblasts. Cells with multiple Auer rods are scarce or absent, but single Auer rods can be found. In contrast to the hypogranular appearance of peripheral blood promyelocytes, the BM promyelocytes may be more typical of the cells found in the hypergranular form of APL. See Appendix B for cytochemical and immunophenotypic results for APL.

A form of APL in which some promyelocytes contain metachromatic granules (when stained with toluidine blue) has been described.[27] Distinctive features include folded nuclei, hypergranular cytoplasm, and coarse metachromatic granules.



■ **FIGURE 26-4** A cell from promyelocytic leukemia. Notice the bundle of Auer rods (Wright-Giemsa stain, 1000× magnification).

Diagnostic Gene Rearrangement. The t(15;17) rearrangement is consistently associated with APL. The break point involves the retinoic acid receptor-α (*RARα*) gene on chromosome 17 and the promyelocytic leukemia (*PML*) gene on chromosome 15 and is limited to the neoplastic cells. Some APL patients have a cytogenetically normal karyotype, but molecular analysis has shown submicroscopic insertion of *RARα* into *PML*, leading to the expression of the PML/RARα transcript. These findings suggest that the t(15;17) translocation/fusion protein is involved in the pathogenesis of the disease. However, other variant translocations have been reported in APL, all involving the *RARα* gene translocated with different target genes, including promyelocytic zinc finger (*PLZF*) on chromosome 11(t[11;17][9q23;q21]), nuclear mitotic apparatus (*NuMA*) on chromosome 15 (t[11;17][q13;q21]), nucleophosmin (*NPM1*) on chromosome 5 (t[5;17][q23;q12]), and signal transducer and activator of transcription 5b (*STAT5b*) on chromosome 17 (t[17;17]).[28]

The typical t(15;17) gene rearrangement results in a fusion *PML/RARα* gene and a reciprocal *RARα/PML* gene. The PML/RARα mRNA has been identified in all APL patients and the reverse translocation RARα/PML mRNA is found in about 75% of APL patients.[29] Molecular analysis of the *PML/RARα* gene is important in monitoring therapy and relapse because the gene disappears with successful treatment but returns as an early marker of relapse. Also, some patients have molecular evidence of the fusion protein product (fusion gene) in the absence of a detectable cytogenetic abnormality.

The RARα protein is a nuclear hormone receptor that binds to specific DNA sequences (RA-responsive elements) and controls transcription of specific target genes under the control of retinoid hormones. RARα forms a complex with a second protein, retinoid-X receptor (RXRα). In the absence of RA, the RARα/RXRα complex represses transcription by recruiting corepressor proteins and inducing histone deacetylation.[27] Physiologic concentrations of RA

normally induce a conformational change in the retinoic acid receptors, causing release of the corepressor molecules and recruitment of coactivator molecules, resulting in gene transcription and granulocytic differentiation.[30]

PML is a growth suppressor nuclear protein normally found in complex macromolecular structures containing numerous other nuclear proteins. The PML/RARα fusion protein binds to the RA-responsive elements of the target genes. Like the native protein, the fusion protein recruits corepressor molecules and in the absence of RA inhibits transcription of RA-responsive genes. Although the fusion protein can bind RA, physiologic concentrations of RA are not sufficient to induce the release of the corepressor proteins from PML/RARα and repression of gene transcription is maintained.[30] Thus, the cells do not differentiate into granulocytes.

Pharmacological concentrations of RA (all-transretinoic acid [ATRA]) have proven to be an effective treatment for inducing complete hematologic remission in APL (but the remission is generally short lived). ATRA induces the neoplastic promyelocytes to differentiate to mature granulocytes, thus overcoming the maturation arrest.[31] It is thought that a high concentration of RA, as is given in induction therapy for APL, overcomes the interference with receptor activation (it promotes release of the corepressor molecules and assembly of the coactivator molecules on the target genes, allowing gene transcription and cellular differentiation). Cells previously unable to mature and initiate apoptosis are then able to do so, causing a transitory situation known as *hyperleukocytosis*.

Unfortunately, the stem cells bearing the t(15;17) translocation are unaffected by ATRA and continue to proliferate, resulting in relapse of the disease. The current approach to therapy is to induce hematologic remission with ATRA and then administer traditional chemotherapy (to try to eradicate the leukemic stem cells). When the promyelocytes are first induced to differentiate and are no longer present in large numbers, the subsequent chemotherapy does not result in the massive death of promyelocytes (and the release of procoagulants), thus avoiding or minimizing the life-threatening effects of DIC. Instead, the chemotherapy destroys the dividing stem cells that bear the t(15;17) translocation and induces a durable remission.[1]

About 25% of patients given RA therapy become acutely ill associated with the death and lysis of the large number of cells. The mortality rate in these individuals can be high. The illness is similar to capillary leak syndrome with fever, respiratory disease, renal impairment, and hemorrhage.[32]

☑ CHECKPOINT 26-4

Why is it important to perform molecular studies on patients with AML with t(15;17)?

Acute Myeloid Leukemia with t(6;9)(p23;q34); *DEK-NUP214*

This AML presents with or without monocytic features often associated with basophilia and multilineage dysplasia. It is detected in 0.7–1.8% of AML and occurs in both children and adults.[10] AML with t(6;9)(p23;q34) usually presents with anemia and thrombocytopenia, and often with pancytopenia. In adults, the white blood cell count is generally lower than other AML categories.[33] Morphologically, they most commonly present with AML with maturation or acute myelomonocytic leukemia. Auer rods are present in one-third of cases. Marrow and peripheral blood basophilia is seen in 44–62% of cases. Most cases show evidence of granulocytic and erythroid dysplasia.

The t(6;9)(p23;q34) results in a fusion of the *DEK* gene on chromosome 6 with the *NUP214* (formerly known as *CAN*) gene on chromosome 9. This results in a nucleoporin fusion protein that acts as an aberrant transcription factor and alters nuclear transport by binding to soluble transport factors.[34] The t(6;9) is the sole abnormality in the majority of cases, but some patients have the t(6;9) in association with a complex karyotype.

Acute Myeloid Leukemia with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*

This AML that can present de novo or arise from a prior MDS. It is often associated with normal or elevated peripheral blood platelet count and has increased atypical megakaryocytes with mono- or bilobated nuclei and associated multilineage dysplasia. AML with inv(3) accounts for 1–2% of all AML.[35] It occurs most commonly in adults with no sex predilection. Patients present with anemia and a normal or elevated platelet count. Some present with hepatosplenomegaly. A subset of cases may have <20% blasts at the time of diagnosis with features of chronic myelomonocytic leukemia. Marrow eosinophils, basophils, and mast cells may be increased. The BM shows small hypolobated megakaryocytes.

A variety of abnormalities in the long arm of chromosome 3 occur in myeloid malignancies; inv(3)(q21;q26.2) and t(3;3)(q21;q26.2) are the most common. The t(3;3)(q21;q26.2) involves the oncogene *EVI1* at 3q26.2 or its longer form *MDS1-EVI1* and *RPN1* at 3q21. *RPN1* may act as an enhancer of *EVI1* expression resulting in increased cell proliferation and impaired cell differentiation. It induces hematopoietic cell transformation.[36] Secondary karyotypic abnormalities are common with inv(3)(q21;q26.2) and t(3;3)(q21;q26.2) with monosomy 7 most common, occurring in approximately 50% of cases, followed by 5q deletions and complex karyotypes.

AML with t(1;22)(p13;q13); *RBM15-MKL1*

This AML generally shows maturation in the megakaryocytic lineage. It is uncommon, representing <1% of all cases. It occurs de novo and is restricted to infants and young children with most cases occurring in the first 6 months of life. The clinical presentation is marked organomegaly, especially hepatosplenomegaly. Patients present with anemia and thrombocytopenia with a moderately elevated white blood cell count. The blasts are similar to those of acute megakaryoblastic leukemia. Small and large megakaryoblasts may be present along with morphologically undifferentiated blasts resembling lymphoblasts. The megakaryoblasts are usually medium to large in size with an indented nucleus, fine reticular chromatin, and one to three nucleoli. The cytoplasm is basophilic and often agranular and may show distinct blebs or pseudopod formation. Micromegakaryocytes are common. Dysplasia in the erythroid and granulocytic cell lines is usually not present.

The t(1;22)(p13;q13) translocation results in the fusion of the RNA-binding motif protein-15 (*RBM15*) gene (1p13) with the megakaryoblastic leukemia-1 (*MKL1*) gene (22q13) and ultimately, expression of the RBM15–MKL1 fusion protein. In most cases, this is the sole abnormality.

The function of *RBM15* is not well defined. It appears to act as a transcriptional repressor that negatively regulates megakaryocyte development. When *RBM15* is lost or nonfunctional, megakaryocytes are increased in number in the BM and spleen.[37] *MKL1* encodes a transcriptional coactivator of genes involved in megakaryocyte differentiation and maturation.[38] The precise role of the fusion protein in AML is unclear; however, it may modulate chromatin organization (epigenetic control) in regions important for megakaryocytic development.[39]

AML with Gene Mutations

Specific gene mutations that do not result in an abnormal karyotype also occur in AML. This includes fms related tyrosine kinase 3 (*FLT3*), nucleophosmin (*NPM1*), and less commonly, mutations of the *CEBPA* gene encoding the CCAAT/enhancer binding protein-α. Cases of AML with these mutations have consistent morphologic, immunophenotypic, and clinical characteristics.

FLT3 and AML

The *FLT3* gene is located at 13q12 and encodes a tyrosine kinase receptor involved in hematopoietic stem cell differentiation and proliferation. The FLT3 protein is expressed on progenitor cells and blasts in most cases of AML. *FLT3* mutations may occur with any AML type and in MDS and can occur with or without additional cytogenetic abnormalities.[33] Mutations lead to constitutively activated FLT3 that transduces signals for cell growth and inhibition of apoptosis.

The two primary types of *FLT3* mutations are internal tandem duplication (*FLT3*-ITD) seen in 30–40% of cases, and mutations affecting the second tyrosine kinase domain (TKD).[5] *FLT3*-ITD mutations are associated with an adverse outcome, but the *FLT3*-TKD mutations remain controversial.[10] Although *FLT3* mutations are not included as a defining criterion for WHO classification, they are important for prognosis and should be evaluated.[5]

AML with Mutated *NPM1*

This form of AML usually involves mutations in exon 12 of the *NPM1* gene. The NPM protein is a chaperone of proteins that shuttles between the nucleus and cytoplasm. *NPM1* gene mutations appear to cause loss-of-function (Chapter 23) of the NPM protein to the extent that NPM remains in the cytoplasm and can no longer translocate to the nucleus.[40]

This AML frequently has myelomonocytic or monocytic features and presents de novo in older adults with a normal karyotype. Usually there is no history of a prior MDS or MPN. Patients often have anemia and thrombocytopenia and a higher white blood cell count than other AML types. There is a slight female predominance. Patients can show extramedullary involvement with the most frequently affected sites being the gingiva, lymph nodes, and skin.[41] *NPM1* mutation is found in approximately 35% of AML patients.[40] *NPM1* mutations can also be seen in AML with and without maturation and in acute erythroid leukemia.

The diagnosis of AML with mutated *NPM1* is made based on the identification of the mutation by molecular studies and is usually associated with a normal karyotype; 5–15% show chromosomal abnormalities including trisomy 8 and del(9q).

AML with Mutated *CEBPA*

Acute myeloid leukemia with mutated *CEBPA* is usually seen in AML with or without maturation. Some cases can show myelomonocytic or monoblastic features. The CEBPA protein is normally expressed at low levels in HSCs, increases in concentration as cells mature to the CMP stage, and decreases again as cells mature to terminally differentiated neutrophils or monocytes. Loss of *CEBPA* gene expression leads to maturation arrest at the CMP.[42]

CEBPA mutations usually present de novo, and are seen in 5–15% of AML.[42] There is no age or sex difference. Patients present with a higher hemoglobin level and lower platelet counts, lower LD levels, and higher peripheral blood blast count when compared with *CEBPA* nonmutated AML. AML with a normal karyotype and *CEBPA* mutation is associated with a favorable prognosis.[40]

☑ CHECKPOINT 26-5

Would the *FLT3*, *NPM1*, and *CEBPA* genes be classified as tumor suppressor or proto-oncogenes?

Therapy-Related Myeloid Neoplasms

This category includes therapy-related acute myeloid leukemia (t-AML), myelodysplastic syndrome (t-MDS), and myelodysplastic/myeloproliferative neoplasms (t-MDS/MPNs). In these cases, AML occurs as a late complication of cytotoxic chemotherapy and/or radiation therapy administered for a prior malignancy. This group of neoplasms is best considered together as a unique clinical syndrome.

Therapy-related neoplasms are thought to be the consequence of mutations induced by cytotoxic therapy. Cytotoxic agents commonly implicated include alkylating agents, topoisomerase II inhibitors, and ionizing radiation therapy. Two subsets are recognized. The most common occurs 5–10 years after receiving the alkylating drug or radiation. The patient often presents with t-MDS, and a minority will present with t-MDS/MPN or t-AML. Patients commonly have an unbalanced loss of genetic material involving chromosome 5 and/or 7. In the second subset, the latency period is shorter, about 1–5 years and follows treatment with topoisomerase II inhibitors. Most of the patients in this category present with acute leukemia.

Approximately 70% of patients have unbalanced chromosomal losses, mainly with chromosome 5 and/or 7, and are often associated with additional abnormalities including del(13q), del(20q), del(11q), del(3p), −17, −18, −21, +8. This type of AML generally has a poor prognosis.[1]

AML with Myelodysplasia-Related Changes

This category is an acute leukemia with morphological features of a myelodysplastic neoplasm, a prior history of myelodysplastic neoplasm, or a myelodysplastic/myeloproliferative neoplasm or an MDS-related cytogenetic abnormality. To classify as AML with myelodysplasia-related changes by morphology, the dysplasia must be present in 50% of cells in at least two cell lines. Dyserythropoiesis is characterized by megaloblastosis, karyorrhexis, nuclear irregularity, and fragmentation, multinucleation, ring sideroblasts, and cytoplasmic vacuoles. Dysgranulopoiesis is characterized by neutrophils with

hypogranular cytoplasm, hyposegmentation (pseudo–Pelger-Huët anomaly), or abnormal segmentation. Dysmegakaryopoiesis is characterized by micromegakaryocytes, megakaryocytes with non-lobated nuclei, or multiple nuclei. The chromosomal abnormalities found in this category are similar to those found in MDS and often involve complex karyotypes with −7/del(7q) and −5/del(5q) being the most common (Table 26-3). Trisomy 8 and del(20q) are common in MDS but are not considered to be disease-specific abnormalities and would not be sufficient by themselves to be considered a case of AML with myelodysplasia-related changes.

Acute Myeloid Leukemias Not Otherwise Specified

This AML category includes cases of AML that do not fulfill criteria for any of the other described groups.[5] The subcategories in this group are primarily differentiated on morphology and cytochemical features. The defining criterion is ≥20% blasts (the blast percentage includes promyelocytes in APL and promonocytes in AML with monocytic differentiation). For pure erythroid leukemias, the blast percentage is based on the percentage of abnormal erythroblasts. Immunophenotypic and cytochemical results for this category of AML are included in Table 26-3.

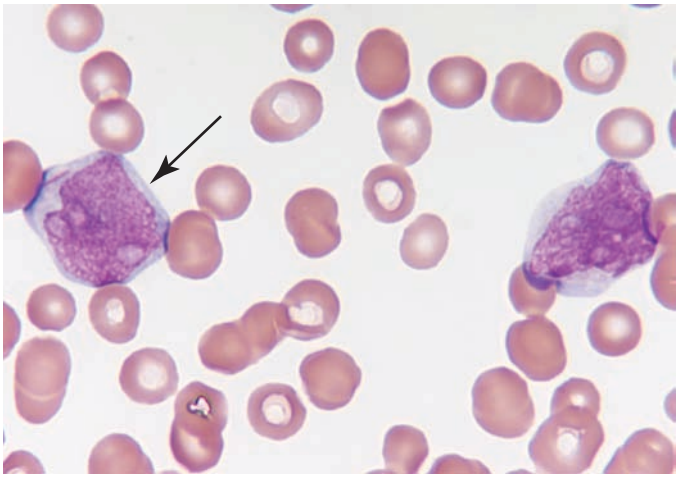
AML with Minimal Differentiation

AML with minimal differentiation is a rather rare leukemia (<5% of all AML cases) and is characterized by lack of evidence of myeloid differentiation by morphology (absence of granules) or cytochemistry (<3% of the blasts are positive for SBB or MPO).[43] This subtype is generally associated with a poor prognosis. The blasts, however, demonstrate myeloid differentiation by flow cytometry for the myeloid lineage (CD13 and CD33). Most cases are also CD34, CD38, and HLA-DR+. No unique chromosomal abnormalities are associated with this subtype. The most common previously reported abnormalities are complex karyotypes and unbalanced abnormalities such as −5/del(5q), −7/del(7q), +8, and del(11q), but the presence of these abnormalities now places them in a different category.[10] Because the blasts in the minimally differentiated AML group have no morphologic differentiating features, immunophenotyping also should be used to exclude the lymphoid lineage. The cell of origin is thought to be a hematopoietic precursor cell at the earliest stage of myeloid differentiation.[44]

AML without Maturation

Characterized by a high percentage of BM blasts without significant evidence of maturation to more mature neutrophils, this variant AML without maturation can occur at any age but most commonly in adulthood. It accounts for 5–10% of the cases of AML. They usually present with BM failure; however, there can be a leukocytosis with markedly increased blasts. The defining feature is its lack of cellular maturation; <10% of all granulocytic cells show evidence of maturation beyond the myeloblast stage (Figure 26-5 ■).

The predominant cell in the peripheral blood is usually a poorly differentiated myeloblast with fine lacy chromatin and nucleoli. Myeloblasts are usually ≥90% of nonerythroid cells in the BM with >3% of blasts positive for MPO and/or SBB. The blasts may have azurophilic granules, Auer rods, and vacuoles. If no evidence of granules or Auer rods is present, the blasts can resemble lymphoblasts and must be differentiated by immunophenotype. Cytochemical staining reactions for Auer rods are similar to reactions for myeloblasts (Table 26-5 ★). There is no demonstrated association between AML without maturation and AML with specific chromosomal abnormalities.



■ **FIGURE 26-5** Peripheral blood film from a patient with AML. The large mononuclear cells are myeloblasts. The cell at the left (arrow) is a myeloblast with an Auer rod. Note the high nuclear-to-cytoplasmic ratio, the fine lacy chromatin, and the prominent nucleoli (Wright-Giemsa stain, 1000× magnification).

☑ CHECKPOINT 26-6

What hematologic features help distinguish AML minimally differentiated from AML without maturation?

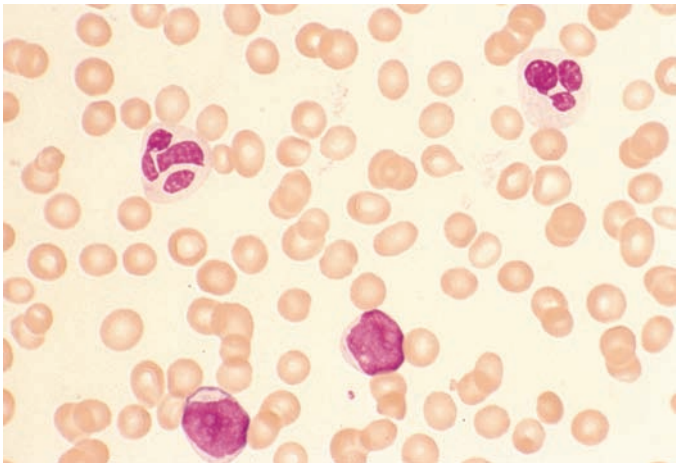
AML with Maturation

AML with maturation is characterized by ≥20% blasts with evidence of maturation to more mature neutrophils (>10% of cells at differentiated stages of maturation: promyelocytes, myelocytes, metamyelocytes). Monocytes and their precursors constitute <20% of the marrow cells. This type of AML occurs in all age groups (20% of patients are <25 years of age, and 45% are >60 years of age) and accounts for about 10% of AML cases.[44] Patients often present with anemia, thrombocytopenia, and neutropenia.

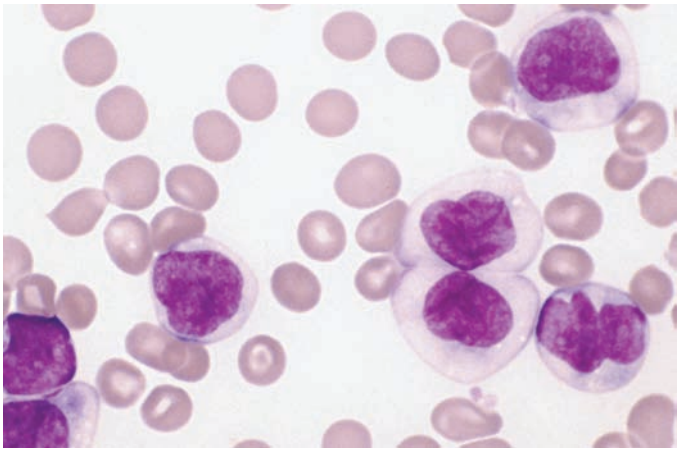
Blasts with and without azurophilic granulation can be present, and blasts frequently contain Auer rods. Variable dysplasia, including myeloid hypogranulation, nuclear hypo- and hypersegmentation, and occasionally binucleated myeloid cells, can be seen (Figure 26-6 ■).

★ **TABLE 26-5** Cytochemical Reactions for Auer Rods

SBB	+
Myeloperoxidase	+
Napthol AS-D chloroacetate esterase	±
PAS	±
Romanowsky	+ or − (occasionally seen only with MPO or SBB)



■ **FIGURE 26-6** Peripheral blood film from a patient with AML with maturation. Myeloblasts are at the bottom and hypogranulated segmented neutrophils are at the top (Wright-Giemsa stain; 1000× magnification).



■ **FIGURE 26-7** Peripheral blood film from a case of acute myelomonocytic leukemia (AMML). Monoblasts and promonocytes are present (Wright-Giemsa stain, 1000× magnification).

The BM is hypercellular, and myeloblasts make up 20–89% of the nonerythroid nucleated cells. Eosinophils and sometimes basophils can be increased. No demonstrated association between AML with maturation and specific recurrent chromosomal abnormalities exists.

☑ CHECKPOINT 26-7

A patient with AML has a peripheral blood differential that includes 91% myeloblasts, 4% promyelocytes, 3% granulocytes, and 2% monocytes, and 30% of the blasts are positive with MPO. Which category of AML is the most likely diagnosis? Explain.

Acute Myelomonocytic Leukemia (AMML)

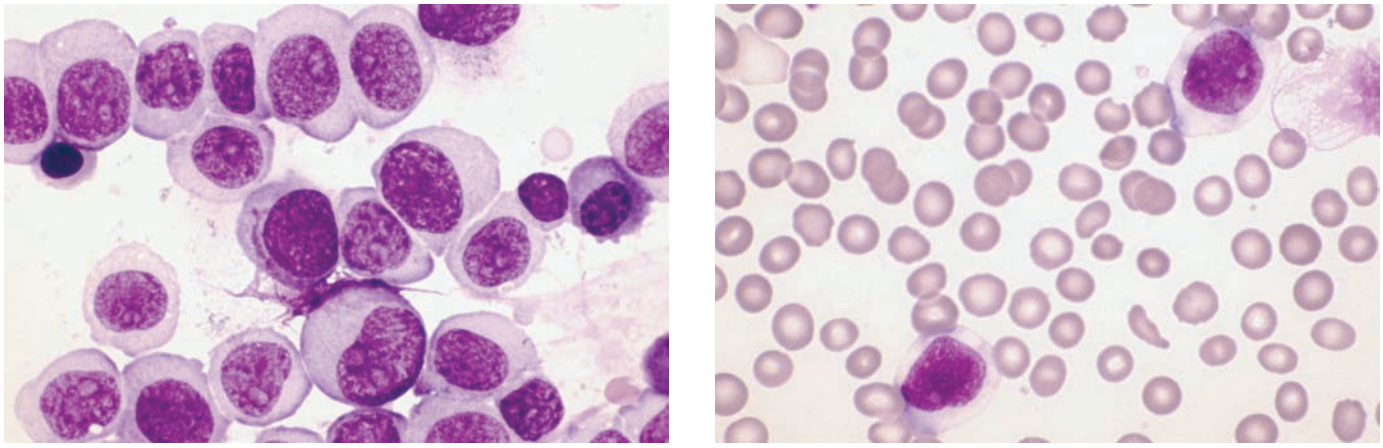
Characterized by proliferation of both myelocytic and monocytic precursors, the BM in acute myelomonocytic leukemia (AMML) is hypercellular with ≥20% blasts; neutrophils and their precursors as well as monocytes and their precursors each compose ≥20% of marrow cells. AMML accounts for 5–10% of AML cases. Some patients have a history of chronic myelomonocytic leukemia. Infiltrations of leukemic cells in extramedullary sites are more frequent than in the pure granulocytic variants. Serum and urinary levels of muramidase (lysozyme) are usually elevated because of the monocytic proliferation.

The peripheral blood leukocyte count in AMML is usually increased. Monocytic cells (monoblasts, promonocytes, monocytes) are increased to $\geq 5 \times 10^9$/L (Figure 26-7 ■). Anemia and thrombocytopenia are present in almost all cases. The myeloblasts appear similar to blasts in AML with differentiation. Monoblasts are usually large with abundant bluish-gray cytoplasm and can show pseudopods. Scattered fine azurophilic granules and vacuoles can be present. The nucleus is round or convoluted with delicate chromatin and one or more prominent nucleoli. The monoblasts and myeloblasts demonstrate the expected cytochemical and immunophenotypic results for their respective lineages (Table 26-5). The BM can reveal erythrophagocytosis by monocytes. The cell of origin is thought to be a hematopoietic precursor cell with the potential to differentiate into neutrophil and monocytic lineages (GMP).[44]

Additional laboratory testing is required when (1) the BM findings are as previously described, but the peripheral blood monocyte count is $< 5 \times 10^9$/L or (2) the peripheral blood monocyte count is $\geq 5 \times 10^9$/L, but the BM has <20% monocytic cells. In these cases, ancillary laboratory tests such as lysozyme levels or cytochemical methods can be utilized to confirm the presence of a significant monocytic component and establish a diagnosis of AMML (Table 26-6 ★).

★ **TABLE 26-6** Nonimmunophenotypic Criteria for Diagnosis of AMML

1. Bone marrow	Blasts ≥20% of nonerythroid cells
	Monocytic cells ≥20% of nonerythroid cells
	Granulocytic cells ≥20% of nonerythroid cells
and peripheral blood	$\geq 5 \times 10^9$/L monocytic cells
or if peripheral blood	$< 5 \times 10^9$/L monocytic cells
Requires ancillary tests	Serum or urinary lysozyme 3× normal
	or naphthol AS-D chloroacetate esterase *and* α-naphthyl acetate esterase (+) in blasts
	or naphthol AS-D acetate esterase with and without NaFl reveal >20% monocytic cells in bone marrow
2. Peripheral blood	$\geq 5 \times 10^9$/L monocytic cells
and bone marrow	Blasts ≥20% of nonerythroid cells
	Monocytic cells <20% of nonerythroid cells
Requires ancillary tests	Serum or urinary lysozyme 3× normal
	or naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase (+) in blasts
	or naphthol AS-D acetate esterase with and without NaFl reveal >20% monocytic cells in bone marrow



a **b**

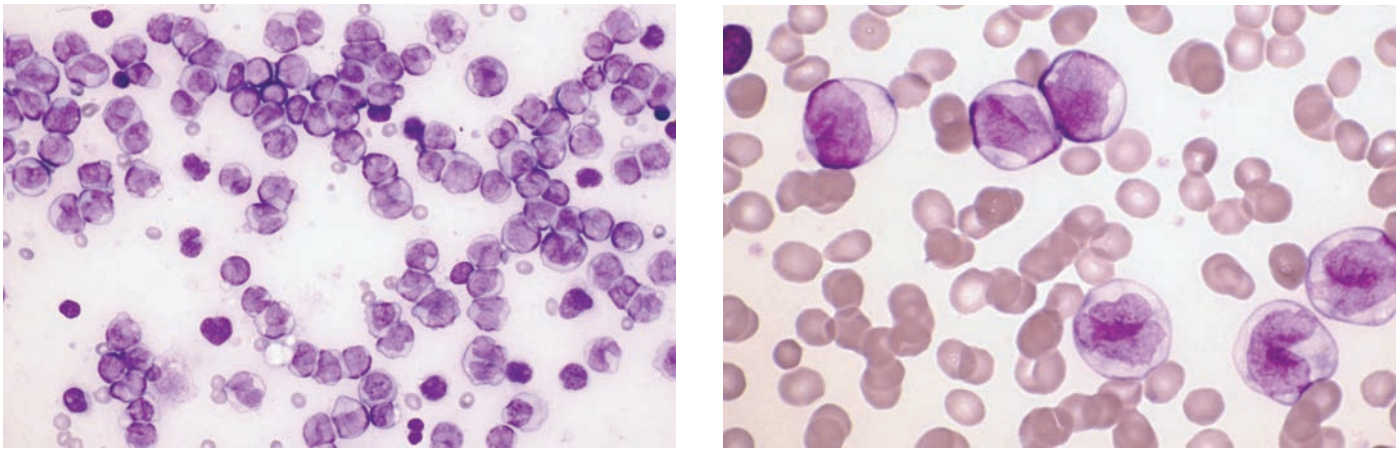
■ **FIGURE 26-8** **(a)** A smear from a bone marrow aspirate of a patient with acute monoblastic leukemia. The cells are predominately monoblasts and promonocytes. **(b)** Monoblasts in peripheral blood from a case of acute monoblastic leukemia (Wright-Giemsa stain, 1000× magnification).

Acute Monoblastic Leukemia and Acute Monocytic Leukemia (AMoL)

These subtypes are myeloid leukemias in which ≥80% of the leukemic cells are monocytic (including monoblasts, promonocytes, monocytes). The neutrophilic component, if present, is <20% of the cells. The majority of the monocytic cells (usually >80%) found in acute monoblastic leukemia are monoblasts (Figure 26-8 ■). The majority of the monocytic cells in acute monocytic leukemia are promonocytes (Figure 26-9 ■). Acute monoblastic leukemia accounts for 5–8% of AML cases, and acute monocytic leukemia accounts for 3–6% of them.[44] This disease is usually seen in children or young adults.

The most common clinical findings are weakness, bleeding, and a diffuse erythematous skin rash. One notable aspect of this disease is the degree of extramedullary leukemic proliferation (gingival and cutaneous infiltration, central nervous system involvement). As with AMML, serum and urine muramidase are moderately elevated. Monocytic cells account for 80% or more of cells in the bone marrow. Monocytes in the peripheral blood are increased, and monoblasts are often present. The monoblasts are large (up to 40 mcM) with abundant, variably basophilic cytoplasm. Pseudopods with translucent cytoplasm are common, and fine azurophilic granules can be present. The nucleus is round or oval with delicate chromatin and one or more prominent nucleoli, but Auer rods are usually not found. Dyshematopoiesis is not conspicuous. Promonocytes have a more irregular and convoluted nucleus, and nucleoli can be present. The cytoplasm is less basophilic than that of the monoblast with a ground glass appearance. Fine azurophilic granules are often present.

Abnormalities of the long arm of chromosome 11 with translocations or deletions are often found in monocytic leukemias. The t(8;16) abnormality is also found and is associated with hemophagocytosis. The expression of *c-FOS* proto-oncogene on chromosome 14 appears to be enhanced in acute leukemias with monocytic lineage



a **b**

■ **FIGURE 26-9** **(a)** A smear from a bone marrow aspirate of a patient with acute monocytic leukemia. There is a predominance of promonocytes and monoblasts (Wright-Giemsa stain, 1000× magnification). **(b)** Monocytic cells in peripheral blood, including promonocytes and monoblasts, from acute monocytic leukemia (Wright-Giemsa stain; 1000× magnification).

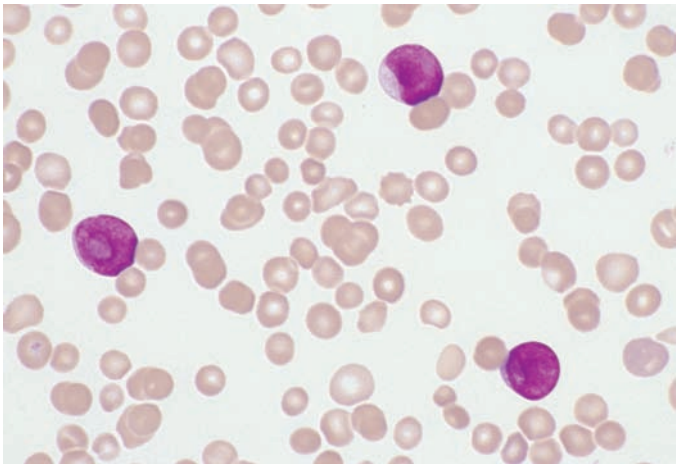
involvement.[1] This gene has been linked to normal monocyte–macrophage differentiation. The cell of origin for this AML subtype is believed to be a hematopoietic precursor cell committed to monocytic differentiation (CFU-M).[44]

Acute Erythroid Leukemia (AEL)

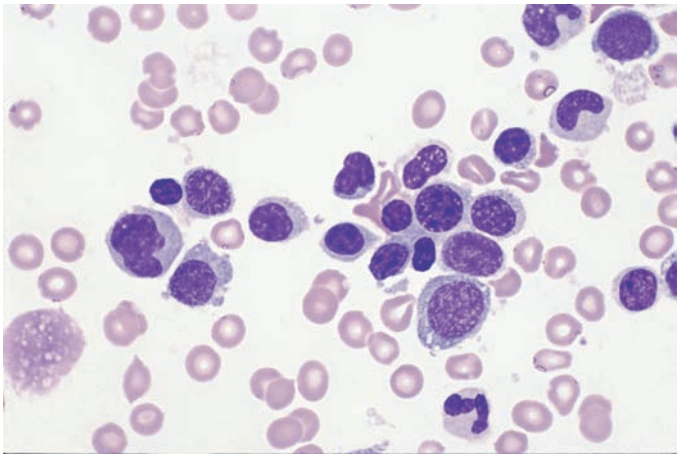
Acute erythroid leukemias (AELs) account for <5% of cases of AML and have a predominant erythroid population. Two variants that are differentiated by the presence or absence of a significant myeloid component are described: the erythroid/myeloid variant and the pure erythroid variant (Figure 26-2). The erythroid/myeloid variant (mixed lineage variant) is defined by ≥50% erythroid precursors in the BM (percent of all nucleated cells) with ≥20% myeloblasts in the nonerythroid cell population. On the other hand, pure erythroid leukemia is an undifferentiated variant that shows neoplastic proliferation exclusively in the erythroid lineage (>80% of total marrow cells are committed to the erythroid lineage).[45] Erythroid/myeloid leukemia is predominantly a disease of adults and accounts for 5–6% of AML cases. Pure erythroid leukemia is extremely rare and can occur at any age.[44]

The most dominant changes in the peripheral blood are anemia with striking poikilocytosis and anisocytosis with a large number of nucleated erythrocytes that are dysplastic with megaloblastoid nuclei and/or bi- or multinucleated cells in the more immature stages. The cytoplasm frequently contains vacuoles. Myeloblasts can be seen in the peripheral blood in the erythroid/myeloid form of AEL (Figure 26-10 ■).

The BM erythroblasts are distinctly abnormal with bizarre morphological features. Giant multilobular or multinucleated forms are common. Other features include nuclear budding and fragmentation, cytoplasmic vacuoles, Howell-Jolly bodies, ringed sideroblasts, and megaloblastoid changes (Figure 26-11 ■). Erythrophagocytosis of the abnormal erythroblasts is a common finding. Auer rods can be found in the myeloblasts in the mixed lineage variant. Dysmegakaryopoiesis is common with mononuclear forms or micromegakaryoblasts present. Neutrophils can exhibit hypogranularity and pseudo–Pelger-Huët anomaly. The leukocyte alkaline phosphatase score is normal or increased. Leukocytes and platelets are usually decreased.



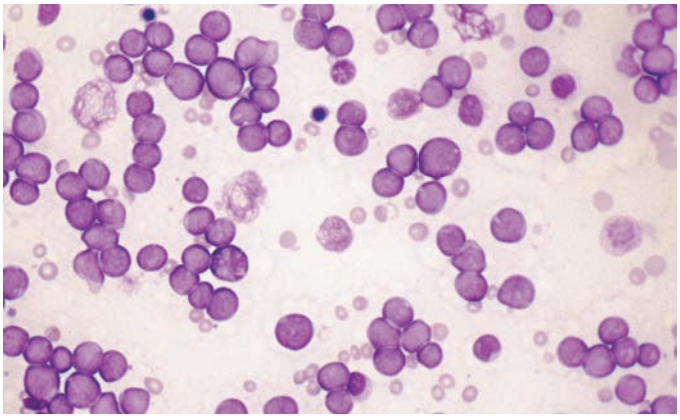
■ **FIGURE 26-10** Myeloblasts in the peripheral blood from a patient with the erythroid-myeloid variant of AEL. The blast morphology is typical of AML without differentiation (Wright-Giemsa stain; 1000× magnification).



■ **FIGURE 26-11** Erythroblasts with megaloblastoid features in the bone marrow from a case of AEL (Wright-Giemsa stain, 1000× magnification).

Normoblasts are typically periodic acid-Schiff (PAS) negative; however, in erythroleukemia, erythroblasts can demonstrate coarse positivity in either a diffuse or granular pattern. PAS-positive erythroblasts are occasionally also found in MDS, other subgroups of AML, iron-deficiency anemia, thalassemia, severe hemolytic anemia, and sometimes in megaloblastic anemia. The myeloblastic component shows reactions similar to those found in other subtypes of AML in both morphology and chromosomal aberrations. Erythroblasts usually react positively with antibodies to glycophorin A (CD71) or hemoglobin A.

The mixed lineage variant occasionally can evolve to a predominantly myeloblastic leukemia (Figure 26-12 ■). The cell of origin for the mixed lineage variant is thought to be a multipotential progenitor cell with wide myeloid differentiation potential. The cell of origin for pure erythroid leukemia is thought to be a primitive progenitor cell committed to the erythroid lineage (BFU-E or CFU-E).[44]



■ **FIGURE 26-12** Smear of a bone marrow aspirate from a patient with erythroid/myeloid AEL that is evolving to a myeloblastic leukemia. Most cells are myeloblasts (Wright-Giemsa stain, 1000× magnification).

☑ CHECKPOINT 26-8

What differentiating hematologic feature of acute erythroid leukemia causes it to be classified into two subgroups?

Acute Megakaryoblastic Leukemia (AMkL)

The megakaryoblastic subgroup of AML is an acute leukemia in which ≥50% of the leukemic blasts are of the megakaryocytic lineage. It occurs in both adults and children and constitutes <5% of all AML cases.[44] This category excludes AML with myelodysplasia-related changes, AML with t(1;22)(p13;q13), inv(3)(q21;q26), t(3;3)(q21;q26.2), and Down syndrome-related cases.

Patients usually present with cytopenias; although most have thrombocytopenia, some can have thrombocytosis. Dysplastic features in the neutrophils and platelets can be present. There is no significant organomegaly. The BM megakaryoblasts are usually medium to large (12–18 mcM) with basophilic, often agranular, cytoplasm. The cells frequently show distinct pseudopod formation. Small blasts resembling lymphoblasts can also be present. On careful examination of the peripheral blood smear, circulating micromegakaryocytes and undifferentiated blasts can be found. However, the finding of cytoplasmic blebs suggests that these cells are megakaryocytic. Megakaryocytic fragments also can be present.

BM aspiration often results in a dry tap because of extensive marrow fibrosis associated with an expanded megakaryocyte lineage. In these cases, marrow biopsy may be required and usually reveals increased fibroblasts and/or increased reticulin as well as >20% blasts. It has been suggested that megakaryocytes secrete a number of mitogenic factors that stimulate fibroblast proliferation.

Blasts can be identified as megakaryocytic by immunophenotyping flow cytometry for CD41, CD61, occasionally CD42, and cytochemistry. The blasts are highly variable, ranging from small round cells with scant cytoplasm and dense heavy chromatin to cells with moderately abundant cytoplasm with or without granules and nuclei with lacy chromatin and prominent nucleoli. Some blasts can have cytoplasmic blebs. Megakaryocytes with shedding platelets occasionally are present. Dysplasia of all cell lines is a common finding.

No unique chromosomal abnormality is associated with acute megakaryoblastic leukemia in adults.

Acute Basophilic Leukemia (ABL)

The primary differentiation of this acute leukemia is along the basophil lineage. Some cases develop as a blast transformation phase of CML. This is a very rare form of AML with few reported cases (<1% of all AML cases). The most characteristic feature by cytochemistry is metachromatic positivity with toluidine blue.[44] The blasts can show diffuse staining with acid phosphatase and occasionally PAS positivity. They are usually negative for typical cytochemical stains for myeloid cells but express myeloid markers (e.g., CD13, CD33). No consistent chromosomal abnormality has been identified with this subtype of AML. The cell of origin is believed to be a myeloid precursor committed to the basophil lineage. The differential diagnosis includes a blast phase of a myeloproliferative neoplasm and other AML with basophilia such as AML with t(6;9)(p23;q34).

Acute Panmyelosis with Myelofibrosis

This disorder is associated with an acute panmyeloid proliferation and fibrosis of the bone marrow. A rare form of AML, it occurs primarily in adults. The major differential diagnosis is with acute megakaryoblastic leukemia (with fibrosis) or other types of AML with associated marrow fibrosis and/or with chronic idiopathic myelofibrosis. This diagnosis is indicated when the proliferative process involves all major myeloid lineages (granulocytes, erythroid cells, and megakaryocytes [i.e., panmyelosis]). Dysplastic, small megakaryocytes are characteristic. The number of blasts varies but is usually between 20–25%. Diagnosis requires BM biopsy and immunohistology.

Myeloid Sarcoma

This designation refers to a disease process in which a tumor mass of myeloblasts or immature myeloid cells occurs in an extramedullary site or in bone. It can occur concurrently with acute or chronic myeloid leukemias or other myeloproliferative or myelodysplastic disorders. The immunophenotype and cytochemistry reflect the specific myeloid cell(s) involved in the malignant process (i.e., myeloblasts, promyelocytes, occasionally monocytes). The cell of origin is believed to be the CMP.

Myeloid Proliferations Related to Down Syndrome

Individuals with Down syndrome have been found to have an increased risk of leukemia, estimated at 10- to 100-fold. They also are at risk for developing transient abnormal myelopoiesis (TAM), which is a unique disorder of Down syndrome. The newborn with TAM presents with clinical and morphologic findings indistinguishable from AML. The blasts are of the megakaryocytic lineage. At presentation, thrombocytopenia is the most common feature. There may be a marked leukocytosis with an increase in blasts. Hepatosplenomegaly may be present. In the majority of cases, spontaneous remission occurs within the first three months of life. In most cases, the blasts are positive for CD34, CD56, CD117, CD13, CD33, CD7, CD4, CD41, CD42, and CD61. In addition to trisomy 21, acquired GATA1 mutations are present in blast cells of TAM. Children with Down syndrome have a 50-fold increase in incidence of acute leukemia during the first 5 years of life. About 1–2% of children with Down syndrome will develop AML during this time. The BM core may show an increase in reticulin fibrosis resulting in a dry tap. The blasts are positive for the same markers as in TAM except that 50% of cases are negative for CD34, and 30% of cases are negative for CD56. In addition to trisomy 21, somatic mutations of the gene encoding the transcription factor GATA1 are considered pathognomonic of TAM or MDS/AML of Down syndrome.

Blastic Plasmacytoid Dendritic Cell Neoplasm

This is a rare, clinically aggressive hematopoietic neoplasm derived from precursors of plasmacytoid dendritic cells that are also known as *type 1 interferon-producing cells*. It has a high frequency of cutaneous and BM involvement and leukemic dissemination. The patients present with skin lesions that can be nodules, plaques, or bruiselike areas. At presentation, regional adenopathy is common, but peripheral blood and BM involvement can be minimal. With disease progression, blood and BM exhibit disease involvement. Following an initial response to chemotherapy, patients variably relapse with involvement in the skin or in other sites including the CNS. The blasts are positive for CD4, CD43, CD56, CD123, TCL1, and CLA. Fifty percent of cases

are positive for CD68. The blasts are negative for alpha-naphthyl-butyrate esterase and MPO. Two-thirds of patients have an abnormal karyotype. Complex karyotypes are common.

CASE STUDY (continued from page 505)

A BM aspirate and biopsy were performed on Guillermo to aid in diagnosis. The BM biopsy revealed a hypercellular marrow. The BM aspirate revealed an M:E ratio of 7.8:1. The predominant cells were abnormal and immature with an indented or lobulated nuclear configuration. Heavy cytoplasmic granulation was present, and multiple Auer rods were in several cells. The cells were strongly positive with MPO.

5. Based on the morphology and cytochemical staining of these cells, what is the most likely AML classification?
6. What is the major complication associated with this leukemia?
7. What chromosome abnormality is associated with this leukemia?

CASE STUDY

8. If this patient were treated with all transretinoic acid, what would you expect to find two weeks later when the blood count was repeated?

THERAPY

Traditional chemotherapy for AML is designed to reduce tumor load as rapidly as possible. Newer treatment modalities include molecularly targeted therapies (e.g., ATRA), epigenetic-targeted therapies (demethylating agents, histone deacetylase inhibitors), and autologous or allogeneic BM transplants and infusions of donor lymphocyte cells with total body irradiation to increase leukemic cell destruction. Current research is focusing on novel therapies including the use of monoclonal antibodies and gene therapy and the destruction of the cellular matrix that supports the neoplastic tissue.

Evaluation of peripheral blood counts is essential to support patients during chemotherapy. Development of at-risk stages such as pancytopenia, severe granulocytopenia, and/or thrombocytopenia must be monitored so that early intervention can occur (growth factors to stimulate hematopoietic cell recovery and/or transfusions).

BM transplantation (Chapter 29), whether allogeneic or autologous, remains the only therapeutic choice that currently provides the potential for a prolonged (10+ years) disease-free survival for most patients with AML.

☑ CHECKPOINT 26-9

Predict the peripheral blood picture of a patient on antifolate chemotherapy.

Summary

The ALs compose a heterogeneous group of neoplastic stem cell disorders characterized by unregulated proliferation and blocked maturation. The two major groups of AL are AML and ALL, which the 2008 WHO Classification System further categorizes into subtypes based on morphologic criteria, cytochemical stains, immunologic analysis, and cytogenetic and molecular abnormalities. The 2008 WHO classification system recognizes AML subtypes with recurrent chromosomal abnormalities, with multilineage dysplasia, and following cytotoxic therapy. It replaces the FAB classification system that was based on morphology and cytochemistry. The WHO system also recognizes new subcategories of acute basophilic leukemia, acute panmyelosis with myelofibrosis, myeloid sarcoma, blastic plasmacytoid dendritic cell neoplasm, and myeloid proliferations related to Down syndrome.

MPO and/or SBB cytochemical staining help differentiate AML (MPO+) from ALL (MPO−). Subgrouping the AMLs requires additional cytochemical stains, immunophenotyping, and cytogenetic analysis. The AML cases that are known as *AML not otherwise specified* are primarily classified by morphology and cytochemistry.

The onset of AML is usually abrupt; without treatment, it progresses rapidly. Symptoms are related to anemia, thrombocytopenia, and/or neutropenia. Splenomegaly, hepatomegaly, and lymphadenopathy are common findings. Hematologic findings include a macrocytic or normocytic, normochromic anemia, thrombocytopenia, and a decreased, normal, or increased leukocyte count. Blasts are almost always found in the peripheral blood. A BM examination is always indicated if leukemia is suspected. The BM reveals ≥20% blasts.

Review Questions

Level I

1. A differential report notes the presence of >20% blasts. This number supports the diagnosis of: (Objective 1)
   A. CML
   B. AML
   C. CLL
   D. MDS
2. The presence of blasts with no evidence of myeloid differentiation by morphology and <3% positive for SBB or MPO is commonly seen in: (Objective 2)
   A. AML—minimally differentiated
   B. AML—without maturation
   C. acute basophilic leukemia
   D. myeloid sarcoma
3. Which M:E ratio is most characteristic of acute leukemia ? (Objective 4)
   A. 1:1
   B. 10:1
   C. 1:5
   D. 4:1
4. A large cell with an immature nucleus containing multiple prominent nucleoli and Auer rods is a(n): (Objective 3)
   A. erythroblast
   B. myeloblast
   C. promyelocyte
   D. promyelocyte with hypergranulation
5. The leukemia that belongs to the WHO classification of AML with recurrent genetic abnormalities is: (Objective 2)
   A. APL
   B. megakaryoblastic leukemia
   C. erythroleukemia
   D. AML minimally differentiated
6. Which of the following subcategories of AML give a positive result with PAS? (Objective 5)
   A. AML without maturation
   B. Acute basophilic leukemia
   C. Acute erythroid leukemia
   D. AML with minimal differentiation
7. Auer rods have been determined to be: (Objective 6)
   A. important to exclude ALL
   B. composed of histamine
   C. positive for proteolytic esterases
   D. present in high numbers in adolescent AML
8. A patient who had been treated previously for myelodysplasia developed AML. What subgroup is most appropriate for a diagnosis in this case? (Objective 2)
   A. AML with recurrent genetic abnormalities
   B. AML with MDS-related changes
   C. t-AML
   D. AML with differentiation
9. The minimum number of blasts in the peripheral blood or bone marrow needed for a diagnosis of AML is: (Objectives 1, 3)
   A. 30%
   B. 10%
   C. 50%
   D. 20%
10. Circulating micromegakaryocytes typically can be found in which of the following acute leukemias? (Objectives 2, 3)
    A. AML with t(8;21)
    B. APL
    C. AML therapy related
    D. acute megakaryoblastic

Level II

1. In attempting to subtype a case of acute leukemia, a laboratory professional noted that the blasts were negative with myeloperoxidase, SBB, specific and nonspecific esterase but positive when stained with PAS and platelet peroxidase. These blasts should show positivity with which of the following monoclonal antibody(ies)? (Objectives 2, 4)
   A. CD41, CD42, CD61
   B. CD24
   C. CD7
   D. glycophorin A
2. The WHO classification of AML is based on: (Objectives 2, 7)
   A. genetic abnormalities
   B. morphology and cytochemistry of blasts
   C. immunophenotyping of blasts
   D. all of the above

3. Which of the following leukemias is associated with cells containing an abundance of Auer rods occurring in bundles? (Objective 2)
   A. APL
   B. AML with multilineage dysplasia
   C. AML t(8;21)
   D. AML therapy related
4. Which of these leukemic blasts demonstrate t(1;22)(p13;q13) positivity? (Objective 4)
   A. erythroblasts
   B. myeloblasts
   C. megakaryoblasts
   D. monoblasts
5. A bone marrow from a 20-year-old male revealed 40% agranular blasts. Cytochemical stains were negative for MPO, SBB, and specific and nonspecific esterases. What immunophenotype panel should be used to help identify the blast cell lineage? (Objective 6)
   A. CD13, CD33, CD34, CD10, CD19, CD2, CD3, CD5
   B. CD11a, CD14, CD10, CD2, CD3, CD71, CD61
   C. CD13, CD33, CD34, CD11b, CD71, CD41
   D. CD41, CD61, CD13, CD15, CD38, CD117
6. A patient has a leukocyte count of 75×10^9/L. A large number of blast cells are present in the peripheral blood smear. Cytochemical stain reveals positive staining with SBB. Immunophenotyping is CD13+, CD33+, CD19−, CD22−, CD3−, CD5−. Karyotyping reveals t(8;21)(q22;q22). Based on the results, these cells would be considered: (Objective 4)
   A. myeloblasts
   B. stem cells
   C. monoblasts
   D. promyelocytes
7. A patient with leukemia is receiving chemotherapy including a drug that is an antagonist to folic acid metabolism. Which of the following types of erythrocytes will be produced? (Objective 5)
   A. macrocytes
   B. microcytes
   C. spherocytes
   D. codocytes
8. Hypergranular promyelocytic leukemia is characterized by which cytogenetic abnormality? (Objective 2,4)
   A. t(15;17)
   B. 11q23 abnormality
   C. t(8;21)
   D. inv(16)(p13;q22)
9. The presence of CD34, CD13, and CD14 is typically associated with a positive: (Objective 4)
   A. PAS stain
   B. TdT
   C. CD71
   D. myeloperoxidase
10. All transretinoic acid therapy is used in leukemia patients with this genetic abnormality: (Objectives 3, 5)
   A. t(9;22)
   B. t(8;21)
   C. trisomy 8
   D. t(15;17)

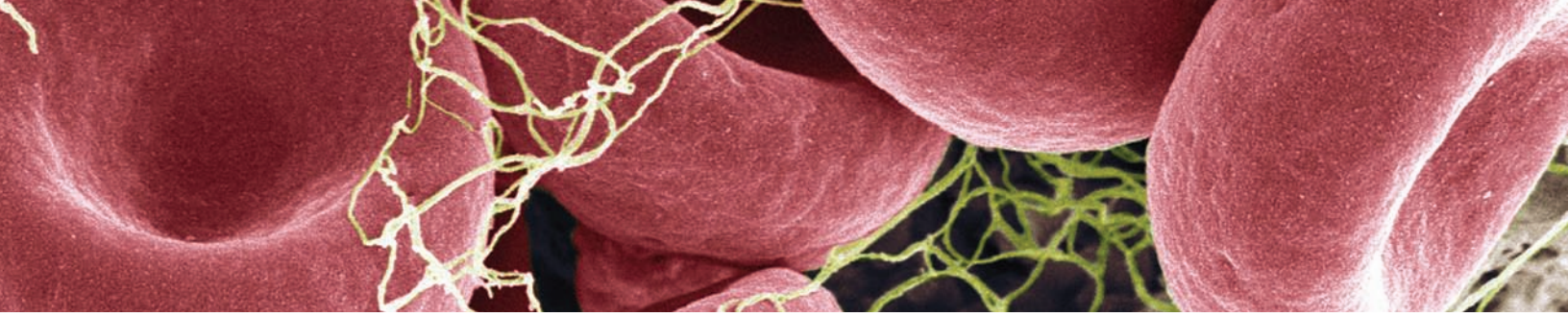
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Liesveld JL, Lichtman MA. Chronic myelogenous leukemia and related disorders. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1277–330.
2. Lechner K, Geissler K, Jager U et al. Treatment of acute leukemia. *Ann Oncol*. 1999;10(suppl 6):45–51.
3. Bennett JM, Catovsky D, Daniel MT et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976;33:451–58.
4. Harris NL, Jaffe ES, Diebold J et al. The World Health Organization Classification of neoplastic diseases of the hematopoietic and lymphoid tissues. *Ann Oncol*. 1999;10:1419–432.
5. Vardiman JW, Thiele J, Arber DA et al. The 2008 revision of the World Health Organization (WHO) classification of the myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–51.
6. Orazi A. Histopathology in the diagnosis and classification of acute myeloid leukemia, myelodysplastic syndromes, and myelodysplastic/myeloproliferative diseases. *Pathobiology*. 2007;74:97–114.
7. First MIC Cooperative Study Group. Morphologic, immunologic, and cytogenetic (MIC) working classification of acute lymphoblastic leukemias: report of the workshop held in Leuven, Belgium, April 22–23, 1985. *Cancer Genet Cytogenet*. 1986;23:189–97.
8. Second MIC Cooperative Study Group. Morphologic, immunologic, and cytogenetic (MIC) working classification of the acute myeloid leukaemias. *Br J Haematol*. 1988;68:487–94.
9. Harris NL, Jaffe ES, Diebold J et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November 1997. *J Clin Oncol*. 1999;17:3835–49.
10. Swerdlow S, Campo E, Harris NL et al., eds. *World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2008.
11. Kheiri SA, MacKerrell T, Bonagura VR et al. Flow cytometry with or without cytochemistry for the diagnosis of acute leukemias? *Cytometry*. 1998;15;34:82–86.
12. Krober SM, Horny HP, Steinke B et al. Adult hypocellular acute leukaemia with lymphoid differentiation. *Leuk Lymphoma*. 2003;44:1797–801.
13. Haferlach T, Bacher U, Kern W et al. Diagnostic pathways in acute leukemias: a proposal for a multimodal approach. *Ann Hematol*. 2007;86:311–27.
14. Kaleem Z, Crawford E, Pathan MH et al. Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. *Arch Pathol Lab Med*. 2003; 127:42–48.
15. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood*. 2008;111:3941–967.
16. Reikvam H, Hatfield KJ, Kittang AO et al. Acute myeloid leukemia with the t(8;21) translocation: clinical consequences and biological implications. *J Biomed Biotechnol*. 2011;2011:104631.
17. Ptasinska A, Assi SA, Mannari D et al. Depletion of RUNX1/ETO in t(8;21) AML cells leads to genome-wide changes in chromatin structure and transcription factor binding. *Leukemia*. 2012;26:1829–41.
18. Goyama S, Schibler J, Cunningham L et al. Transcription factor RUNX1 promotes survival of acute myeloid leukemia cells. *J Clin Invest*. 2013;123:3876–88.
19. Mrozek K, Bloomfield CD. Clinical significance of the most common chromosome translocations in adult acute myeloid leukemia. *J Natl Cancer Inst Monogr*. 2008;39:52–57.
20. Reilly JT. Pathogenesis of acute myeloid leukaemia and inv(16)(p13;q22): a paradigm for understanding leukaemogenesis? *Br J Haematol*. 2005;128:18–34.
21. Muntean AG, Hess JL. The pathogenesis of mixed-lineage leukemia. *Annu Rev Pathol*. 2012;7:283–301.
22. Deschler B, Lubbert M. Acute myeloid leukemia: epidemiology and etiology. *Cancer*. 2006;107:2099–107.
23. Meijers JC, Oudijk EJ, Mosnier LO et al. Reduced activity of TAFI (thrombin-activatable fibrinolysis inhibitor) in acute promyelocytic leukaemia. *Br J Haematol*. 2000;108:518–23.
24. Lemez P. A case of acute promyelocytic leukaemia with "faggot cells" exhibiting strong alpha-naphthylbutyrate esterase activity. *Br J Haematol*. 1988;68:138–39.
25. Castoldi GL, Liso V, Specchia G et al. Acute promyelocytic leukemia: morphological aspects. *Leukemia*. 1994;8:1441–46.
26. Bennett JM, Catovsky D, Daniel MT et al. A variant form of hypergranular promyelocytic leukaemia (M3). *Br J Haematol*. 1980;44:169–70.
27. Gianni M, Ponzanelli I, Mologni L et al. Retinoid-dependent growth inhibition, differentiation and apoptosis in acute promyelocytic leukemia cells: expression and activation of caspases. *Cell Death Differ*. 2000;7:447–60.
28. Redner RL. Variations on a theme: the alternate translocations in APL. *Leukemia*. 2002;16:1927–32.
29. Walz C, Grimwade D, Saussele S et al. Atypical mRNA fusions in PML-RARA positive, RARA-PML negative acute promyelocytic leukemia. *Genes Chromosomes Cancer*. 2010;49:471–79.
30. Grignani F, DeMattels S, Nervi C et al. Fusion proteins of the retinoic acid receptor-a recruit histone deacetylase in promyelocytic leukemia. *Nature*. 1998;391:815–18.
31. Tomita A, Kiyoi H, Naoe T. Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As2O 3) in acute promyelocytic leukemia. *Int J Hematol*. 2013;97:717–25.
32. Tallman MS, Andersen JW, Schiffer CA et al. Clinical description of 44 patients with acute promyelocytic leukemia who developed the retinoic acid syndrome. *Blood*. 2000; 95:90–95.
33. Slovak ML, Gundacker H, Bloomfield CD et al. A retrospective study of 69 patients with t(6;9)(p23;q24) AML emphasizes the need for a prospective, multicenter initiative for rare poor prognosis myeloid malignancies. *Leukemia*. 2006; 20:1295–97.
34. Scandura JM, Bocccuni P, Cammenga J et al. Transcription factor fusions in acute leukemia: variations on a theme. *Oncogene*. 2002;21:3422–444.
35. Byrd JC, Mrozek K, Dodge RK et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia; results from Cancer and Leukemia Group B (CALGB 8461). *Blood*. 2002;100:4325–36.
36. Lahortiga I, Vazquez I, Agirre X et al. Molecular heterogeneity in AML/MDS patients with 3q21q26 rearrangements. *Genes Chromosomes Cancer*. 2004;40:179–89.
37. Raffel GD, Mercher T, Shigematsu H et al. Ott1(Rbm15) has pleiotropic roles in hematopoietic development. *Proc Natl Acad Sci U S A*. 2007;104:6001–6.
38. Cheng EC, Luo Q, Bruscia EM et al. Role for MKL1 in megakaryocytic maturation. *Blood*. 2009;113:2826–34.
39. Lee JH, Skalnik DG. Rbm15-Mkl1 interacts with the Setd1b histone H3-Lys4 methyltransferase via a SPOC domain that is required for cytokine-independent proliferation. *PLoS One*. 2012;7:e42965.
40. Falini B, Mecucci C, Tiacci E et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005;352:254–66.
41. Verhaak RG, Goudswaard CS, van Putten W et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood*. 2005;106:3747–754.
42. Paz-Priel I, Friedman A. C/EBPα dysregulation in AML and ALL. *Crit Rev Oncog*. 2011;16:93–102.
43. Patel KP, Khokhar FA, Muzzafar T et al. TdT expression in acute myeloid leukemia with minimal differentiation is associated with distinctive clinicopathological features and better overall survival following stem cell transplantation. *Mod Pathol*. 2013;26:195–203.
44. Jaffe ES, Harris NL, Stein H et al., eds. *World Health Organization Classification of Tumours*. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon, France: IARC Press; 2001:63–73.
45. Huang Q. Pure erythroid leukemia. *Arch Pathol Lab Med*. 2004;128:241–42.



27 Precursor Lymphoid Neoplasms

SUSAN J. LECLAIR, PHD
GAIL BENTLEY, MD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *acute lymphoblastic leukemia* (*ALL*) and *lymphoblastic lymphoma* (*LBL*), and differentiate them from acute myeloid leukemia (AML).
2. List and define the variants of ALL/LBL according to the WHO classification.
3. Describe and recognize the typical peripheral blood picture (erythrocytes, leukocytes, blasts, thrombocytes) seen in ALL.
4. Give the typical results of flow cytometric analysis and genetic findings in ALL.
5. Summarize the clinical signs and symptoms and the most frequent age groups associated with ALL.
6. Define the rare acute leukemias that are not included in the AML and ALL groups.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Compare and contrast the various presentations of ALL/LBL.
2. Predict the most likely WHO or immunophenotypic subgroup based on patient history, physical assessment, and laboratory findings.
3. Correlate cellular presentation with prognosis and common complications in ALL/LBL.
4. Correlate Wright stained blast morphology in the ALL subgroups with flow cytometry and genetic testing results.
5. Evaluate peripheral blood results in relation to response to oncological therapy (e.g., complete or partial remission, relapse).
6. Identify acute leukemia (AL) from a peripheral blood smear and recommend laboratory tests that may be useful in differentiation of acute leukemia of ambiguous lineage, AML, ALL, and the subgroups of ALL.
7. Define the phases and purposes of chemotherapy for ALL.
8. Correlate clinical and laboratory findings with prognosis in ALL/LBL.
9. Contrast the clinical and laboratory findings of ALL to LBL.

Chapter Outline

Key Terms

Acute undifferentiated leukemia (AUL)
B-cell acute lymphoblastic leukemia
Bilineage acute leukemia
Biphenotypic acute leukemia
Consolidation therapy
Induction therapy
Maintenance chemotherapy
Minimal residual disease (MRD)
T-cell acute lymphoblastic leukemia

Background Basics

The information in this chapter will build upon concepts learned in previous chapters. To maximize your learning experience, you should review and have an understanding of these concepts before starting this unit of study:

Level I

- Summarize the origin and differentiation of hematopoietic cells. (Chapter 4)
- Describe the maturation, differentiation, and function of the lymphocytes. (Chapter 8)
- Outline the classification and general laboratory findings of the acute leukemias. (Chapter 23)
- Summarize the typical laboratory findings that define acute myeloid leukemia. (Chapter 26)

Level II

- Summarize the role of oncogenes and growth factors in cell proliferation, differentiation, and maturation. (Chapters 4, 23)
- Diagram the maturation pathway for T and B lymphocytes. (Chapter 8)
- Describe the role of molecular analysis in diagnosis and treatment of acute leukemia. (Chapter 42)
- Describe the use of immunophenotyping in acute leukemia. (Chapters 23, 40)
- Describe the role of cytogenetics and diagnosis and treatment of acute leukemia. (Chapters 23, 41)

CASE STUDY

We will refer to this case study throughout the chapter.

Dan, a 4-year-old white male, saw his physician for symptoms of easy fatigue and bruising. His mother stated that until one month ago, he was a "typical kid." Since then she has noticed increased lassitude, a regression to more babylike behavior, and loss of appetite. For the past two days, his temperature has been 100°F. Upon physical examination, the child presented as pale, quiet, and of appropriate size for his age. Most systems were unremarkable with the exception of several small firm lymph nodes felt in the cervical and auxiliary regions. His CBC revealed a WBC count of 40.2 $\times$ 10^9/L with 90% blasts.

Consider the laboratory testing that could help in diagnosing Dan's illness.

OVERVIEW

This chapter discusses the acute lymphoblastic leukemias and lymphomas. A summary of clinical signs and symptoms is juxtaposed with the hematologic findings. The 2008 World Health Organization (WHO) Classification System is used. The emphasis is on the laboratory techniques and results used to diagnose and classify the neoplasms. The rare acute leukemias that are not grouped into either acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) are termed *acute leukemias of ambiguous lineage* and also are reviewed. ALL may also be referred to as lymphoblastic leukemia (LL).

INTRODUCTION

Lymphoid malignancies include a wide spectrum of syndromes from disorders that primarily involve the bone marrow and peripheral blood (leukemias) to those that initially present as solid tissue masses (lymphomas) primarily involving lymphoid organs (lymph nodes, tonsils, spleen, thymus, and the lymphoid tissue of the gastrointestinal tract). These lymphoid neoplasms can be composed primarily of mature or precursor lymphoid cells, a distinction that serves as a means for classification. The mature lymphoid leukemias and lymphomas are discussed in Chapter 28. This chapter includes a description of the precursor or immature lymphoid leukemias (ALL) and lymphomas (lymphoblastic lymphoma [LBL]). Although ALL is primarily a neoplasm of the bone marrow and peripheral blood and LBL is a neoplasm that presents as a solid mass in lymphoid tissue, the two entities are not always distinct. Tissue involvement can occur in ALL, and peripheral blood and bone marrow involvement can occur in LBL. It is thought that ALL and LBL are different clinical expressions of the same disease and therefore are grouped together in the WHO classification. They arise from a malignant change in the hematopoietic stem cell, common lymphoid progenitor (CLP), or more differentiated progenitors of the T- or B-cell lineage.

ETIOLOGY AND PATHOGENESIS

Similar to AML (Chapter 26), the ALLs are hematologic disorders characterized by malignant neoplastic proliferation and accumulation of immature and dysfunctional hematopoietic cells in the bone marrow. Multiple somatically acquired mutations within a stem cell prior to its differentiation into a lymphoid progenitor or

within a lymphoid precursor cell gives rise to a clone of malignant lymphocytes. Thus, ALL/LBL can be either T- or B-cell phenotypes. How the cells acquire these mutations is unknown, but a combination of leukemogenic factors could be responsible (Chapter 23). The types of genetic alterations include chromosomal rearrangements and abnormalities of cell ploidy as well as point mutations in oncogenes or tumor suppressor genes that alter cellular functions (Chapter 23). These genetically abnormal cells proliferate in an unregulated manner with altered responses to growth and antigrowth signals and exhibit maturation arrest at any one of several early stages of differentiation. They divide and synthesize DNA more slowly than normal hematopoietic cells, but they have enhanced self-renewal and increased resistance to apoptotic signals.[1] Thus, the leukemic clone expands and the neoplastic cells accumulate. Impairment of normal hematopoiesis as the leukemic clone expands is the primary cause of concern.

CLINICAL FINDINGS

Although ALL has been diagnosed in all age groups, the incidence of the disease demonstrates two clear frequency clusters, one between the ages of 2 and 5 years and another in the sixth decade. In elderly people, the signs and symptoms—complaints of fatigue, infections, and bruising—are typically more acute than in children. The onset of the disease in children can be insidious or abrupt with nonspecific signs and symptoms.

The clinical picture of anemia, increased infections, and thrombocytopenia results from bone marrow failure because of the replacement of normal marrow elements by leukemic lymphoblasts. Pallor, fatigue, and shortness of breath reflect the anemia, which tends to become symptomatic below 10 g/dL of hemoglobin. Fever can result from an infection or remain classified as a fever of unknown origin, perhaps because of the release of endogenous pyrogens and other inflammatory compounds. Both petechiae and ecchymoses are present in >50% of the patients. Bone pain, especially tenderness of the long bones, presents in about 80% of children with ALL, and is often excused as "growing pains" but is actually from the expansion of marrow space by leukemic cells. Weight loss with or without anorexia is common because of the negative nitrogen balance of metabolically abnormal cells. Leukemia infiltrates are responsible for splenomegaly, hepatomegaly, and lymphadenopathy when present.

B-cell ALL is characterized by inappropriate secretion of monoclonal immunoglobulins that can increase blood viscosity and impair blood flow through the microcirculation (hyperviscosity syndrome). These immunoglobulins also can impair granulocyte and platelet function, induce pathologic rouleaux, interact with coagulation proteins, and interfere with coagulation. Production of autoantibodies can lead to autoimmune hemolytic anemia, autoimmune thrombocytopenia, and autoimmune neutropenia. Extramedullary involvement is common, affecting the central nervous system, lymph nodes, spleen, liver, and testis.

The neoplasm can present with primary involvement of nodal and extranodal sites with minimal or no involvement of bone marrow and peripheral blood. The disease is then referred to as *LBL*. Sites of involvement in B-cell LBL include skin, soft tissue, and lymph nodes. In T-cell LBL, mediastinal (thymic) involvement is common. When the patient presents with a neoplastic lymphoid mass and lymphoblasts in the bone marrow, the distinction between lymphoma and leukemia is arbitrary. However, when peripheral blood and bone marrow are extensively involved, the disease is known as *lymphoblastic leukemia*. Without leukemia, LBL is usually asymptomatic.

T-cell ALL and LBL tend to have more aggressive clinical behavior than B-cell malignancies. These T-cell malignancies often involve extranodal and extramedullary sites.[1] Lymphadenopathy and hepatospenomegaly are usually present.

☑ CHECKPOINT 27-1

Compare the typical age groups in which AML and ALL are found.

LABORATORY FINDINGS

Evaluation of the peripheral blood and bone marrow is critical for making a diagnosis of ALL (Table 27-1 ★).

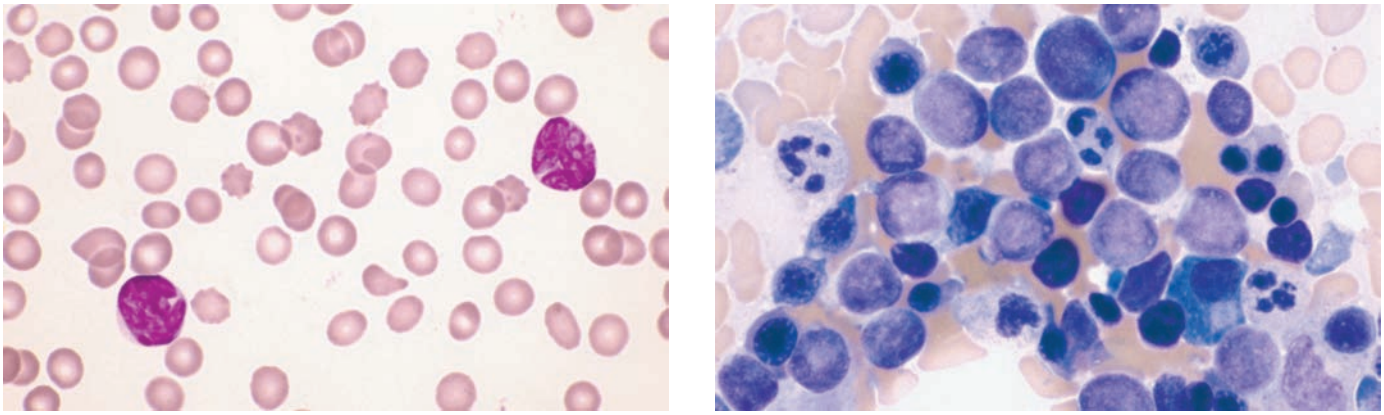
Peripheral Blood

The white blood cell count can be increased, decreased, or within the reference interval.[2,3] Hyperleukocytosis (>100.0 × 10^9/L) occurs in 11–23% of all patients and most commonly in patients with T-cell ALL. The absolute neutrophil count is decreased in 20–40% of patients, even when there is leukocytosis, and correlates strongly with the incidence of infection.[1] Although no established minimum percentage of blasts defines ALL (as there is for AML), WHO recommends that the diagnosis of ALL not be made if there are <20% blasts.[3] Many treatment protocols use the criterion of a minimum of 25% blasts for a diagnosis of ALL.

T and B lymphoblasts are not distinguishable by morphology. Circulating lymphoblasts are found in the peripheral blood in 90% of cases.[1] They vary from small cells with scant to moderate light basophilic or grey-blue cytoplasm, a high N:C ratio, slightly condensed chromatin, and indistinct nucleoli to larger cells with a moderate amount of cytoplasm, finely dispersed chromatin, and distinct nucleoli[1] (Figures 27-1 ■ and 27-2 ■). The nucleus can be irregularly shaped.

★ **TABLE 27-1** Initial Laboratory Findings Characteristic of ALL

Peripheral blood	• Leukocyte count usually increased but can be normal or decreased • Neutropenia • Lymphoblasts present • Normocytic, normochromic anemia • Thrombocytopenia
Bone marrow	• Hypercellular • Increased lymphoblasts, usually >25%, not <20%



a **b**

■ **FIGURE 27-1** **(a)** Lymphoblasts in peripheral blood from acute lymphoblastic leukemia. Notice the nuclear cleavage, condensed chromatin, and high N:C ratio. **(b)** Lymphoblasts in the bone marrow from the patient with ALL in **(a)**. Note cells with a high N:C ratio, fine chromatin, and nucleoli (both Wright-Giemsa stain, 1000× magnification).

The larger blasts may be intermixed with the smaller blasts (Figure 27-2). The cytoplasm can contain vacuoles and amphophilic granules (that stain a pinkish to purple color) that are probably lysosomal in origin.[4] These granules can make it difficult to distinguish these lymphoblasts from myeloblasts.

Unless accompanied by bleeding, a normochromic normocytic anemia is common. Anisocytosis, poikilocytosis, and nucleated RBCs are usually not present.[5] There is typically thrombocytopenia ($48–52 \times 10^9$/L).

CASE STUDY (continued from page 523)

The physician ordered a CBC on Dan. The results were as follows:

		WBC Differential	
WBC	40.2×10^9/L	Lymphoblasts	90%
RBC	3.45×10^{12}/L	Neutrophils	8%
Hb	9.7 g/dL	Monocytes	1%
Hct	0.32 L/L (32%)	Eosinophils	1%
MCV	92.7 fL		
MCH	28.1 pg		
MCHC	30.3		
RDW	17.3		
PLT	63×10^9/L		

On a scan of the peripheral blood smear, rare nucleated erythrocytes were seen, and the platelets appeared decreased in number.

1. Based on these data, what would be the initial interpretation of Dan's presentation?

Bone Marrow

The bone marrow is preferred for diagnosis because there are no circulating blasts at diagnosis in up to 10% of patients with ALL. Marrow cells also are better for genetic studies. The bone marrow in most patients reveals a marrow densely populated with lymphoblasts (>65%), and aspiration may not be possible. In this case, biopsy is indicated.

The morphology of the cells in the marrow mirrors that seen in the peripheral blood. Auer rods are not present in lymphoblasts; if Auer rods are seen, the blasts are distinguished as myeloblasts.

Tissue Involvement

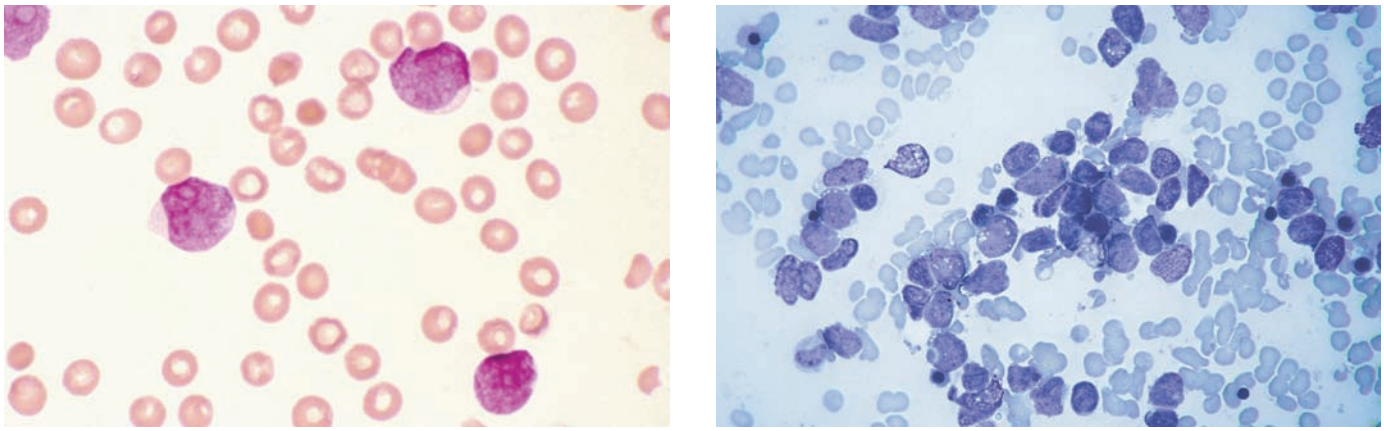
Lymphoma is the term used to describe lymphoid neoplasms when there is mass lesion of lymphoid or other tissue with no or little involvement of the bone marrow and peripheral blood. In B-LBL, lymph nodes and other involved tissue show diffuse or paracortical patterns of infiltration by malignant lymphoid cells. There are usually numerous mitotic figures. There may be a focal "starry sky" pattern due to macrophage phagocytosis of apoptotic tumor cells. In T-LBL, lymph nodes can show total architectural effacement that involves the capsule. Sometimes the involvement in the paracortical area is only partial, sparing the germinal center. A multinodular pattern and "starry sky" effect can be present.

Other Laboratory Findings

As with other leukemias, other laboratory findings are consistent with increased cellular metabolism and, in general, the extent of abnormality of the various laboratory parameters is proportional to the tumor burden. Hyperuricemia and an increased lactate dehydrogenase are common expressions of cell turnover. Hypercalcemia can occur, which is thought to be from bone resorption associated with leukemic infiltration in the bone marrow. The typical consequence of leukemic infiltration of the kidney is impaired renal function with increased serum creatinine, phosphorus, blood urea nitrogen, and uric acid. Because the central nervous system (CNS) is a frequent site for extramedullary metastasis of ALL, cerebrospinal fluid analysis may be used throughout treatment to investigate the possibility of leukemic infiltration.

☑ CHECKPOINT 27-2

Explain the difference between ALL and LBL.



a b

■ **FIGURE 27-2** **(a)** Lymphoblasts in peripheral blood from acute lymphoblastic leukemia. Note the fine chromatin and nucleoli (Wright-Giemsa stain, 1000× magnification). **(b)** Lymphoblasts in the bone marrow from patient in **(a)**. Note the blasts with vacuoles (Wright-Giemsa stain, 500× magnification)

IDENTIFICATION OF CELL LINEAGE

The first step in classifying acute leukemia (AL) is to differentiate ALL from AML, usually by identifying the lineage of the blasts. Although cell morphology on Romanowsky-stained smears and cytochemistry can help distinguish ALL from AML, classification of ALL/LBL into subgroups relies on immunologic, cytogenetic, and molecular genetic methods.

Immunophenotyping is used to determine the cells' lineage (myeloid or lymphoid), subtype (T or B cell), and maturation stage. Cytogenetics and molecular analysis can provide evidence of clonality, reveal distinct genetic abnormalities associated with subgroups of ALL/LBL, and provide important prognostic information. Identification of these cellular characteristics can reveal the biologic subtypes, which is important for optimizing treatment outcomes.

Morphology and Cytochemistry

Morphology is important since some neoplasms have characteristic or diagnostic cell features. However, differentiation of ALL from AML (AML minimally differentiated or AML without maturation) may not be possible by morphologic examination of Romanowsky-stained smears alone. When morphologic examination cannot differentiate lymphoblasts from other blasts, cytochemistry may be helpful (Chapter 23). In lymphoblasts, the myeloperoxidase (MPO) and Sudan black B (SBB) stains are negative, which are usually sufficient to differentiate lymphoblasts from myeloblasts. The periodic acid-Schiff (PAS) reaction usually demonstrates a coarse granular positivity in lymphoblasts, especially those with a T-cell immunophenotype. The PAS reactivity may be present in AML, but the granular pattern is superimposed on a diffusely positive background, whereas there is no background positivity in lymphoblasts.

Terminal Deoxynucleotidyl Transferase (TdT)

Certain intracellular enzymes are helpful in identifying cellular subtypes. The most important of these is terminal deoxynucleotidyl transferase (TdT), a DNA polymerase found in cell nuclei. Its presence can be determined by direct enzyme assay, by indirect immunofluorescence, or with monoclonal antibodies. This enzyme is not present in normal mature lymphocytes but can be found in 65% of the total thymic population of lymphocytes with the TdT positive cells localized in the cortex.[6] It can also be found in very early B cells and occasionally very early myeloblasts and therefore, about 1–3% of normal bone marrow cells are TdT positive. Its value in ALL is to differentiate early precursor lymphoblasts from more mature cells.

Immunophenotyping

The immunologic phenotype of neoplastic cells is helpful in determining lineage of the neoplastic cells, differentiating a benign from a neoplastic process, classification and prognosis of the disease, and detection of **minimal residual disease (MRD)** after treatment. The appearance of specific cell markers (antigenic determinants) is developmentally regulated in normal lymphoid cells (Chapters 7,8). Some antigenic determinants appear at a very early stage of development and disappear with age, while others appear on mature cells. Studies of surface and intracellular markers reveal that lymphoblasts in ALL share markers with normal lymphoid counterparts in various stages of maturation[7] (Chapter 8). However, immunophenotyping also has demonstrated that while some leukemic cells have phenotypes of normal cells, others can show asynchronous gene expression, resulting in an inappropriate combination of antigens or lineage plasticity[8] (Figure 27-3 ■). Because no one marker is specific for any neoplasm, a panel of antibodies must be used for accurate diagnosis.

Immunophenotyping is important in differentiating ALL from minimally differentiated AML and in identifying subtypes of the neoplastic lymphoblast (T or B). Immunophenotyping also is valuable in recognizing leukemias of mixed lineage. After therapy, immunophenotyping is used in combination with molecular studies (e.g., polymerase chain reaction [PCR]/fluorescence in situ hybridization [FISH], Chapter 42) to determine MRD.

☑ CHECKPOINT 27-3

Why is it necessary to immunophenotype the lymphoblasts in ALL if they have been identified as lymphoblasts morphologically?

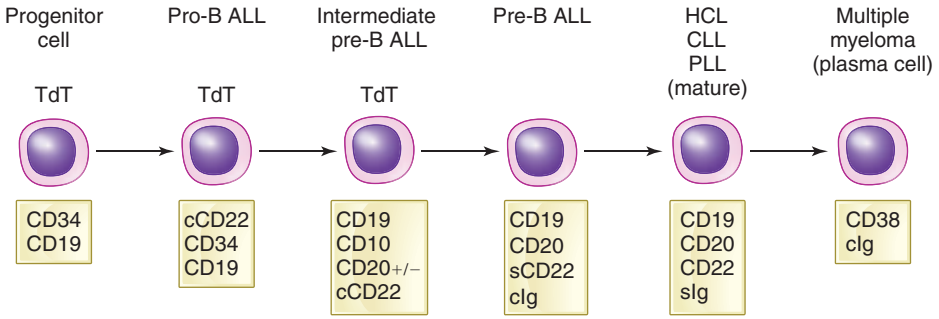


FIGURE 27-3 B lymphocyte maturation pathway with leukemic and other lymphoproliferative counterparts.

ALL = acute lymphoblastic leukemia; CLL = chronic lymphocytic leukemia; PLL = prolymphocytic leukemia; HCL = hairy cell leukemia; clg = cytoplasmic immunoglobulin; slg = surface immunoglobulin

Cytogenetic Analysis

Chromosomal abnormalities are present in 75% of ALL. This information has diagnostic and prognostic implications. Some specific abnormalities are recurrent and used to subgroup the B-cell ALL/LBL.

Molecular Analysis

Molecular analysis can determine the presence of rearrangements of immunoglobin heavy (IGH) chain genes and/or light chain genes and T-cell receptor (TCR) genes (Chapter 8). Rearrangement of IGH chain genes occurs before cellular expression of immunoglobulin (Ig) and is an early genetic marker of B-cell ontogeny.[9] Rearrangement of TCR polypeptide genes is an early sign of T-cell lineage. Molecular analysis of these gene rearrangements can be used to establish clonality of the T- or B-cell populations as well as cell lineage. Clonal gene rearrangement helps to differentiate neoplastic lymphoid cells from normal lymphoid cells. However, IGH gene rearrangement is seen in some instances of T-cell ALL, and TCR gene rearrangement occurs in some cases of B-cell ALL.[9] Thus, identification of additional markers of B- and T-cell lineage commitment is required for diagnosis. Point mutations are less frequently observed but may include genes of major cell-cycle regulator proteins such as p53, various cyclin-dependent kinase inhibitors (CDKi), including p16, p15, and p14, and activating mutations of proto-oncogenes involved in signaling pathways that govern cell proliferation, such as *RAS* (Chapter 2).[10,11]

CASE STUDY *(continued from page 525)*

2. What tests should be used as the initial follow up in this case?

WHO CLASSIFICATION

The WHO Classification defines two subgroups of precursor ALL/LBL: Precursor B-cell and precursor T-cell neoplasms (leukemia/lymphoma) (Table 27-2 ★). These precursor neoplasms with bone marrow and peripheral blood involvement are acute lymphoblastic leukemias, whereas precursor T-cell and precursor B-cell neoplasms presenting as solid tumors are lymphoblastic lymphomas (Chapter 28).

B-cell ALL/LBLs that are characterized by recurrent genetic abnormalities are subgrouped as *ALL with recurrent cytogenetic abnormalities* (see Appendix B for these subgroups and cellular characteristics). The remainder are subgrouped as ALL not otherwise specified (NOS). Although Burkitt lymphoma was classified as ALL in the FAB classification (L3), it is suggested that the B-cell ALL designation not be used to designate Burkitt leukemia/lymphoma.[1] Burkitt lymphoma is included in the WHO classification of mature B-cell neoplasms and is discussed in Chapter 28. There are no recurrent cytogenetic changes observed that are specific to LBL.[9] T-cell ALL has not been subgrouped by recurrent genetic abnormalities as the pathogenesis of most T-cell ALL/LBL is not known.

B Lymphoblastic Leukemia/Lymphoma

Precursor B-cell leukemia (B-cell ALL) is a neoplasm of lymphoblasts committed to the B-cell lineage, which involves the bone marrow and peripheral blood. Occasionally, the disease can present with primary involvement of the lymph nodes or extranodal sites, in which case it is called *B lymphoblastic lymphoma (B-LBL)*. Although arbitrary and with exceptions, if a patient has a mass lesion and 25% or fewer lymphoblasts in the bone marrow, the term *lymphoma* is preferred.

★ **TABLE 27-2** Classification of Lymphoid Leukemia and Lymphoma by the World Health Organization

	Subgroup	Subgroup	Subgroups
B-cell neoplasms	Precursor B-cell neoplasms	Lymphoblastic leukemia Lymphoblastic lymphoma	B-cell ALL/LBL with recurrent genetic abnormalities (see Appendix B) B-cell ALL/LBL not otherwise specified
	Mature B-cell neoplasms	(See Chapter 28)	(See Chapter 28)
T-cell neoplasms	Precursor T-cell neoplasms	Lymphoblastic leukemia Lymphoblastic lymphoma	
	Mature T-cell neoplasms	(See Chapter 28)	(See Chapter 28)

B-cell ALL accounts for 80–85% of the cases of ALL in children and about 70% of the cases of ALL in adults. In general, children diagnosed with precursor B-cell ALL have a good prognosis (long-term event-free survival >80%). Adult event-free survival is lower, 30–50%, depending on the disease subtype and the patient's age at diagnosis (there is an inverse relationship between age and prognosis).

Immunophenotyping

Immunophenotyping can identify markers on neoplastic B cells that correlate to normal stages of maturation within the B-cell lineage (pro-B, intermediate pre-B, and pre-B; see Table 27-3 ★). However, for therapeutic purposes, it is necessary to distinguish only between precursor B-cell, mature B-cell, and T-cell immunophenotypes.[12] It is recommended that panels for cell identification include at least one highly sensitive marker, CD19, and a highly specific marker, cytoplasmic CD79a and CD22, for B-cell lineage.[12]

The earliest stage of differentiation is the pro-B cell and is characterized by an immunophenoptype pattern of TdT+, HLA-DR+, and almost always CD19+ and CD79a+. The intermediate pre-B cell (also known as the *common ALL cell*) expresses CD10 (the common ALL antigen, or CALLA).[13,14] The most mature precursor B-cell differentiation stage is the pre-B cell, which can be CD10 negative but positive for CD20, surface CD22 (sCD22), and cytoplasmic immunoglobulin (clg). However, surface immunoglobulin is absent. Malignancies involving more mature stages of B-cell maturation are discussed in the classification of mature lymphoid neoplasms (Chapter 28).

The majority of ALL/LBL presentations in both children and adults arise from precursor or immature B cells that typically have the same immunophenotype as normal immature B cells (CD10, CD19, CD34, CD22, and TdT). This makes the distinction between normal and neoplastic cells challenging, especially when assessing minimal residual disease unless the abnormal cells have an additional or distinct marker.[15] Up to 50% of B-cell ALL cells coexpress a myeloid associated antigen—CD13, CD15, or C33—that can help in the differentiation between normal and neoplastic. (See Chapter 40 and Appendix B for immunophenotypes associated with specific leukemias.)

Cytogenetics and Pathogenesis

Cytogenetic abnormalities associated with B-cell ALL include translocations, hypodiploidy, and hyperdiploidy.[10] The most significant translocations that have distinctive clinical or phenotypic properties and important prognostic information are[16] (Chapter 24):

- t(12;21)(p13;q22.3)/*ETV6-RUNX1*
- t(4;11)(q21;q23)/*MLL-AFF1*
- t(9;22)(q34;q11)/*BCR-ABL1*
- t(5;14)(q31;q32.3)/*IL3-IGH2*
- t(1;19)(q23.3;p13.3)/*E2A-PBX1(TCF3-PBX1)*

The most common translocation (present in about 25% of cases) in childhood B-cell ALL is t(12;21)(p13;q22.3), producing the *ETV6(TEL)-RUNX1(AML1)* fusion gene.[17,18] Both the *ETV6* and *RUNX1* genes are translocated in other types of leukemia—including t(5;12), t(8;21), and t(3;21)—that are found in various subtypes of AML. The ETV6-RUNX1 fusion protein is believed to initiate and drive leukemogenesis in cells carrying the translocation by disrupting the normal processes of cell differentiation, apoptosis, cell adhesion, and response to DNA damage.[17] Expression of the

★ **TABLE 27-3** Summary of Laboratory Features Helpful in Classification of ALL

Characteristic	Pro-B	Common ALL (intermediate pre-B)	Pre-B	Precursor T
Gene rearrangement				
Immunoglobulin (Ig)	+/−	+	+	−[a]
T-cell receptor (TCR)	−	−	−	+[b]
Immunologic features				
Cytoplasmic Ig	−	+	+	−
Surface Ig	−	−	+/−	−
Immunophenotype				
CD34	+	+/−	−	+
CD19	+	+	+	−
CD22	+(c)	+(c)	+(s)	−
CD10	−	+	+/−	−
CD20	−	+/−	+	−
CD2, CD3, CD5, CD7	−	−	−	+
Cytochemistry				
TdT	+	+	+	+
PAS	−	−	−	+

[a]About 20% of T-ALL exhibits rearrangement of Ig gene
[b] A small number of B-ALL exhibits rearrangement of TCR gene
+ = positive; − = negative; (c) = cytoplasmic; (s) = surface

ETV6-RUNX1 fusion protein in B-cell ALL is associated with an excellent prognosis with event-free survival approaching 90%.

Infants who present with ALL commonly carry the t(4;11)(q21;q23) translocation that fuses the mixed lineage leukemia (*MLL*) gene with the *AFF1* (AF4/FMR2 family, member 1) gene. The MLL protein is involved in chromatin remodeling and epigenetic transcriptional control of cell-cycle regulatory proteins[18] (Chapter 23) whereas the AFF1 protein is important for transcriptional elongation.[19] The MLL/AFF1 fusion protein contributes to uncontrolled proliferation of cells.[18]

The t(9;22)(q34.1;q11.2)/*BCR-ABL1* translocation is more common in adults (10–15%) than children with B-cell ALL.[20,21] In most childhood cases, the *BCR/ABL1* translocation results in a p190 kD fusion protein, whereas in adult cases, about one-half of the translocations produce the p210 kD protein present in CML. The remainder produce the p190 protein (Chapter 24).

The t(5;14)(q31;q32.3) results in a translocation between the *IL3* gene and the *IGH2* gene, which results in a variable eosinophilia that is reactive, not clonal. Blasts are CD19+ and CD10+. The diagnosis can be made on immunophenotypic and genetic information regardless of the blast count in the bone marrow.

Hyperdiploid B-cell ALL is common in children and is seen in 20–26% of B-ALL cases.[22] Almost all cases of hyperdiploid B-cell ALL have mutations in the receptor tyrosine kinase FLT-3, resulting in constitutive activation of the receptor.[23] Hypodiploid clones may also have structural abnormalities but none are specific for diagnosis. Cytogenetic abnormalities considered to be poor prognostic factors are t(4;11)(q21;q23)/*MLL-AFF1* (9% of cases), t(1;19)(q23.3;p13.3)/*PBX1-E2A* (5% of cases), t(9;22)(q34;q11.2)/*BCR-ABL1* (4% of cases), and hypodiploidy (5% of cases).[1,24]

Prognosis

Precursor B-cell ALL generally has a good prognosis. In the pediatric population, the complete remission rate approaches 95%; in adults, it is 60–85%.[25] Long-term, event-free survival is lower for both patient groups (~80% and 30–50%, respectively). Generally, hyperdiploidy (>50 chromosomes) is a better prognostic finding than hypodiploidy (<46 chromosomes) with event-free survival rates nearly 90%. Patients with hyperdiploid chromosome counts <50 have a worse prognosis.[7,8] Positive predictive factors in children include diagnosis at ages 1–9, hyperdiploid chromosomes or t(12;21), and a low or normal white blood cell (WBC) count at diagnosis.[1] Adverse factors include very young age (<1 year), high WBC counts at diagnosis, and other cytogenetic abnormalities listed in "Cytogenetics and Pathogenesis" (Table 27-4 ★).

★ **TABLE 27-4** Poor Prognostic Indicators in ALL

Clinical findings
• Infants (<1 year old)
• Patients beyond puberty
• CNS or mediastinal involvement
Laboratory findings
• High blast counts
• Presence of *BCR-ABL1*

T Lymphoblastic Leukemia/Lymphoma

Accounting for approximately 15% of the cases of childhood ALL and approximately 25% of the cases of adult ALL, precursor T-cell leukemia (T-cell ALL) involves lymphoblasts committed to T-cell lineage in the bone marrow and peripheral blood. If the primary site of involvement is a lymph node or extranodal site, the disease is termed *T lymphoblastic lymphoma (T-LBL)*.

Precursor T-cell ALL usually presents with a high WBC count and often a mediastinal mass (because of leukemic infiltration of the thymus) or other tissue masses. The lymphoblasts are similar to those seen in B-cell ALL, although they are more likely to be variable in size, and cytoplasmic vacuoles can be present. The cytochemistry is also similar to that seen in B-cell ALL, but acid phosphatase can show focal intense positivity in T-cell ALL.

Immunophenotype

The lymphoblasts in T-cell ALL are usually TdT+, CD7+, and CD3+ (Table 27-5 ★). However, only CD3 is considered lineage specific. There is variable expression of CD1, CD2, CD4, CD5, CD8, and CD10.[26] Lymphoblasts frequently coexpress CD4 and CD8, indicative of the cortical stage of thymocyte differentiation ("double-positive cells").[26] At the medullary stage of differentiation, the cells are either CD4+ or CD8+. CD79a, generally considered to be a B-lineage marker, has been observed in some cases. One or more of the myeloid-associated markers (CD13, CD15, CD33) can occasionally be seen. As in B-cell ALL, T-cell ALL also can be stratified into differentiation stages, with cytoplasmic CD3, CD2, and CD7 appearing in the earliest stage followed by CD5, CD1a, and subsequently, the appearance of membrane CD3.[26]

☑ CHECKPOINT 27-4

A patient has 50% blasts in his bone marrow. Immunophenotyping is CD19 positive, but CD20, CD2, CD10, and CD7 negative. What additional testing can be helpful to distinguish the immunologic subgroup of this leukemia?

Cytogenetics and Pathogenesis

T-cell lineage can be determined by detecting rearrangement of the TCR genes using molecular methods. There are four TCR genes that are capable of rearranging and encode the α-, β-, γ-, and δ-chains of the TCR (Chapter 8). Detection of a monoclonal TCR gene rearrangement suggests a neoplasm of the T-cell lineage, but about 20% of T-cell ALL cases exhibit simultaneous rearrangement of one of the immunoglobulin genes. In addition, a few cases of B-cell ALL have shown TCR gene rearrangement.

About one-third of the cases of T-cell ALL have translocations involving the α and δ T-cell receptor loci (14q11.2), the β locus (7q35), or the γ locus (7p14–15). The translocations involve a variety of partner genes, including the transcription factors *MYC* (8q24.1), *TAL1* (1p32), *RBTN1* (11p15), *RBTN2* (11p13), *HOX11* (10q24), and *HOX11L2* (5q35).[27] The result is usually a dysregulation of the partner gene, resulting in growth enhancement. In addition, molecular mutations of the *NOTCH1* gene are found in > 50% of cases of T-cell

★ **TABLE 27-5** Cellular Markers Useful in Diagnosis and Classification of T-Cell ALL

T Cell Developmental Stage	HLA-DR	TdT	CD34	CD2	CDla	CD3	CD7	CD8	CD4	CD10	CD19, CD20, CD22	Ig genes rearrangement	TCR genes rearrangement
Pro-T	+/−	+/−	+/−	−	−	+ (c)	+	−	−	+/−	−	−	+/−
Pre-T	+/−	+/−	+/−	+	−	+ (c)	+	−	−	+/−	−	−	+/−
Cortical T	+/−	+/−	−	+	+	+ (c)	+	+	+	+/−	−	−	+/−
Medullary T	+/−	+/−	−	+	−	+ (c)	+	+[a] or −	+[a] or −	+/−	−	−	+/−

[a]Either CD4 or CD8+

(c) = cytoplasmic; − = absent; + = present; +/− = may be present; Ig = immunoglobulin; TCR = T-cell receptor

ALL.[28] NOTCH1 is a transmembrane receptor that is involved in the regulation of normal early T-cell development. *MYC*, the downstream target of NOTCH1, is involved in the growth of neoplastic cells.

Prognosis

Although acute precursor T-cell leukemia was considered a high-risk disease with a poorer prognosis than B-cell ALL, current therapeutic protocols are improving the prognosis for this disease. T-cell ALL in children increases the risk for induction failure and relapse. In adults, the prognosis of T-cell ALL may be better than of B-cell ALL. This may be related to a lower incidence of adverse cytogenetic mutations in T-cell ALL.

CASE STUDY *(continued from page 527)*

3. If the flow cytometry pattern showed a positive CD10, what would be the classification of this acute leukemia?
4. In this situation, would the therapeutic outcome be considered as favorable or bleak? Why?

☑ CHECKPOINT 27-5

A 3-year-old patient has 45% lymphoblasts in the bone marrow. If the cells tested positive for CD19, CD10, and CD34, what is the most likely immunologic subgroup? Why should cytogenetics be performed on this patient?

Acute Leukemias of Ambiguous Lineage

This WHO group of leukemias include malignant neoplasms in which the morphology, cytochemistry, undefined genetic alterations, and immunophenotype lack sufficient information to classify them within a given lineage or in which the blasts have morphologic and/or immunophenotypic characteristics of more than one lineage.

Natural Killer (NK) Cell Lymphoblastic Leukemia/Lymphoma

Natural killer (NK) cell lymphoblastic leukemia is a neoplasm that is difficult to define because 1) no specific markers for human NK-cell progenitors have been identified and 2) NK-cell progenitors can express markers that are seen in T-cell ALL (CD7, CD2, CD5, cytoplasmic CD3). The CD56 marker was previously thought to identify NK leukemia but many of these cases are now known to be plasmacytoid dendritic cell leukemia. More specific markers for precursor NK cells are CD94 and CD161, but they are not usually tested. Until more specific panels for NK cells are developed, the WHO recommends that NK-cell lymphoblastic leukemia be considered a provisional entity.[29]

Acute Undifferentiated Leukemia

Although determination of the myeloid or lymphoid origin of blasts is important for treatment decisions, doing so is sometimes not possible with current methods. The **acute undifferentiated leukemia (AUL)** category includes acute leukemias in which the morphology, cytochemistry, and immunophenotype of the proliferating blasts lack sufficient information to classify them as either myeloid or lymphoid origin. In this case, the leukemia is classified as acute undifferentiated leukemia.[29,30] Electron microscopic studies can sometimes detect ultrastructural evidence of primary granules and/or peroxidase, a finding that would indicate acute myeloid leukemia. Immunophenotyping should be done with a comprehensive panel of monoclonal antibodies to exclude neoplastic cells of unusual lineages such as basophils and NK-cell precursors. Absence of primary granules and/or MPO with electron microscopy with other negative or ambiguous findings indicates a diagnosis of AUL. It is predominantly diagnosed in adults, and only about one-third of patients respond to the chemotherapy regimens for AML and ALL.

Acute Leukemias with Lineage Heterogeneity

The category acute leukemias with lineage heterogeneity includes those leukemias in which the blasts have morphologic and/or immunophenotypic characteristics of both myeloid and lymphoid cells (or of both T and B lymphocytes). Terminology regarding these disorders has been confusing. If two distinct populations of blasts are identifiable, each expressing markers of a distinct lineage (i.e., one population myeloid and one population lymphoid or one population T lymphoid and one population B lymphoid), the disease has been called **bilineage acute leukemia**. If the blasts coexpress myeloid and T or B lineage-specific markers or concurrent T and B lineage markers, the disease has been called **biphenotypic acute leukemia**.[30] Also recognized is a leukemia with myeloid/natural killer (NK) cell acute leukemia with markers of both NK cells (CD56) and myeloid lineage (CD13/CD33/MPO).[30] WHO classification attempts to clarify the terminology confusion and refers to these biphenotypic

and bilineage leukemias by the general term *mixed phenotype acute leukemia (MPAL)*. The terms *B/myeloid leukemia* and *T/myeloid leukemia* are used to designate leukemias containing the two lineages specified regardless of whether one or two populations of blasts are seen.

The MPAL designation is reserved for cases in which lineage assignment is ambiguous. It should not be used when an ALL aberrantly expresses one or two myeloid antigenic markers or an AML aberrantly expresses one or two lymphoid antigenic markers. These are considered *myeloid antigen–positive ALL* and *lymphoid antigen–positive AML*, respectively. Because many markers are only lineage associated, not lineage specific, coexpression of only one or two cross-lineage antigens is insufficient for a diagnosis of biphenotypic leukemia.

Lack of lineage specificity (lineage infidelity) could be the result of genetic misprogramming, or the leukemic clone could represent a bipotential cell that has retained both lymphoid and myeloid markers during development. Although mixed lineage leukemias are uncommon, their identification is important to determine their actual occurrence, to identify appropriate therapy, and to correlate karyotypic abnormalities.[31,32] A large percentage of the acute leukemias of ambiguous lineage has associated cytogenetic abnormalities. The cell of origin is thought to be the multipotential hematopoietic stem cell.

Current methodology using two or more immunologic markers in a double-labeling technique distinguishes whether the lymphoid and myeloid antigens are on the same cell or separate cells.[29] This can be done with flow cytometry or immunohistochemistry on tissue sections. Another procedure is to stain smears with MPO to identify myeloid cells coupled with flow cytometry to detect the T or B lymphoid population.

☑ CHECKPOINT 27-6

A patient with acute leukemia has two morphologically different types of blasts. One population is positive for CD7 and CD2. The other is positive for CD33 and CD13. What is the most appropriate classification of this leukemia?

THERAPY

New treatment protocols have raised the complete remission rate for B-cell ALL to >95% in children and 60–85% in adults. The prognosis of T-cell ALL/LBL is not as favorable as B-cell ALL/LBL. This may be in part because of the presence of high-risk clinical features (Table 27-4). A significant number of acute leukemias express inappropriate combinations of antigens, making diagnosis challenging. Treatment protocols and prognosis are proving to be more effective and accurate when the leukemic cell lineage is accurately classified immunologically. Therapy response and detection of residual leukemic cells (MRD) is possible using immunophenotyping and genetic testing.

Chemotherapy for ALL is divided into several phases. The **induction therapy** phase is designed to reduce the disease to complete remission (i.e., eradicating the leukemic blast population). This is followed by a CNS prophylactic phase. CNS leukemia is the most common form of relapse in young children who have not undergone specific treatment to the brain and spinal column early in remission. The two potential modes of treatment in the CNS prophylactic phase are cranium irradiation and/or intrathecal chemotherapy. Cranial irradiation is seldom a component of most current treatment protocols because of the risk of neurocognitive deficits and endocrinopathy and of inducing a second cancer.[1] The third phase is **maintenance chemotherapy**, also called *cytoreductive therapy* or remission **consolidation therapy**.[1] The need for this type of therapy is controversial. Some studies have shown a slight increase in survival with its use, whereas others reveal no improvement. Before the institution of CNS prophylactic treatment, there was a high incidence of relapse. The maintenance therapy was designed to prevent this relapse and prolong remission. The purpose of maintenance therapy is to eradicate any remaining leukemic cells. Drug treatment usually continues for 2–3 years. The relapse rate after cessation of all therapy is about 25% in the pediatric patient population.

Allogeneic hematopoietic stem cell transplantation (HSCT) remains controversial. Currently, most clinicians consider it to be of benefit to some high-risk adult patients.[1] Patients who relapse while on therapy or after only a short remission are often considered candidates for HSCT. The use of umbilical cord blood as a source of hematopoietic stem cells is being considered more frequently, especially in the pediatric patient population because it does not require the same degree of histocompatibility as do transplants using peripheral blood or marrow stem cells.

Relapse is defined as the reappearance of leukemic cells anywhere in the body, although the bone marrow is the most common site. Leukemic relapse occasionally occurs at extramedullary sites. Most relapses occur during treatment or within the first two years after completion. Rarely, relapses have been observed up to ten years after the induction of remission. Relapse indicates a poor outcome for most patients, especially if it occurs during therapy or after only a brief initial remission.

Summary

Acute leukemias represent a heterogeneous group of precursor hematopoietic neoplasms characterized by unregulated proliferation, arrested maturation, and/or ineffective apoptosis. The World Health Organization classification categorizes two major groups: precursor (acute) myeloid and precursor (acute) lymphoid. The precursor lymphoid group includes lymphoblastic lymphomas (LBLs) and acute lymphoblastic leukemia (ALL). LBL presents with a mass of malignant lymphoid cells in nodal or extranodal sites with little or no bone marrow and peripheral blood involvement. ALL presents with bone marrow and

peripheral blood involvement. The two groups are considered to be different clinical presentations of the same disease.

ALL/LBL is subdivided into precursor B cell and precursor T cell based on the lineage of the neoplastic blast population. Further subgrouping is based on morphology, genetic or karyotypic mutational status, and immunophenotype of blasts. The MPO and/or SBB can help differentiate AML (peroxidase and SBB positive) from ALL (peroxidase and SBB negative). Various cytogenetic and molecular changes are described in ALL, including t(12;21)(p13;q22)/*ETV6-RUNX1* most commonly and t(9;22)(q34;q11.2)/*BCR-ABL1* that is associated with a poor prognosis.

Regardless of subtype, the onset of ALL is usually abrupt and without treatment, progresses. Symptoms are related to anemia, thrombocytopenia, and/or neutropenia. Splenomegaly, hepatomegaly, and lymphadenopathy are common findings. Hematologic findings of ALL include a normocytic, normochromic anemia, and thrombocytopenia and a decreased, normal, or increased leukocyte count. Blasts are almost always found in the peripheral blood. Although the WHO classification does not require a minimum number of blasts for a diagnosis of ALL/LBL as it does for AML, it is recommended that a diagnosis of ALL not be made for blast counts <20%.

Acute leukemia with lineage heterogeneity includes two situations in which ALs have characteristics of T and B cells or myeloid and lymphoid cells. Mixed phenotype acute leukemia (MPAL) describes ALs with blasts that possess markers of multiple lineages or two different populations of blasts.

Review Questions

Level I

1. Acute lymphoblastic leukemia is characterized by the presence of: (Objective 1)
   A. <20% small resting lymphoblasts in the bone marrow
   B. hypercellular bone marrow with increased lymphoblasts
   C. 30% or more lymphoblasts with erythroid dysplasia in the bone marrow
   D. >30% myeloblasts in the peripheral blood
2. Acute lymphoblastic leukemia is most often seen in patients: (Objective 5)
   A. older than 60 years
   B. between the ages of 35 and 60 years
   C. between the ages of 10 and 35 years
   D. younger than 5 years
3. ALL characterized by the presence of small basophilic lymphoblasts with a round nucleus, finely granular chromatin, and inconspicuous neucleoli can be classified as: (Objective 3)
   A. ALL—Burkitt's type
   B. Acute biphenotypic leukemia
   C. Acute lymphoblastic leukemia
   D. Acute leukemia of ambiguous lineage
4. The presence of CD19 and CD22(c) and absence of CD10 (CALLA) on neoplastic lymphoblasts is most often a sign of: (Objective 4)
   A. biphenotypic leukemia
   B. pro-B ALL
   C. pre-B ALL
   D. T-cell ALL
5. The anemia most often seen in patients with ALL is: (Objective 5)
   A. microcytic hypochromic
   B. normocytic normochromic
   C. macrocytic normochromic
   D. macrocytic hypochromic
6. Leukemia of ambiguous lineage is most often described as the presence of: (Objective 6)
   A. large, basophilic blasts with slight to moderate granulation
   B. blasts demonstrating both CD10 and C33
   C. CD3 and CD4 markers
   D. no demonstrable markers using immunophenotyping
7. The over-secretion of monoclonal immunoglobulins is most often seen in: (Objectives 3, 5)
   A. B-cell ALL
   B. T-cell ALL
   C. B-cell LBL
   D. T-cell LBL
8. CD2, CD3, and CD4 are present on the cells found in a patient with a previously undiagnosed leukemia. These markers are characteristic of: (Objective 4)
   A. pre-B-cell ALL
   B. pro-B-cell ALL
   C. T-cell ALL
   D. ALL—Burkitt's type

9. Acute biphenotypic leukemia is characterized by the presence of: (Objective 6)
   A. ultrastructual evidence of primary granules
   B. CD19, CD20, and CD23
   C. CD2, CD4, CD8, and presence of myeloperoxidase
   D. negative immunophenoptying
10. Which of the following laboratory results are most commonly found in ALL? (Objective 3)
   A. eosinophilia and basophilia
   B. neutropenia and thrombocytopenia
   C. neutrophilia and thrombocytopenia
   D. lymphocytosis and thrombocytosis

Level II

1. Cells that are positive for t(9;22) are characteristic of which of the following? (Objective 4)
   A. T-cell ALL
   B. reactive lymphocytosis
   C. acute undifferentiated leukemia
   D. B-cell ALL
2. Monoclonal rearrangement of the TCR genes is associated with blasts that have the following immunophenotype: (Objective 2)
   A. CD2+, CD4+, CD19+
   B. CD19+, CD20+, CD10+
   C. CD3+, CD13−, CD34+
   D. CD2+ CD3+, CD4+
3. A peripheral blood smear is noted to have 80% blasts. There are two different types of blast cells present: one that is small, with a round nucleus, indistinct nucleoli, and scanty cytoplasm while the second is a large blast with basophilic cytoplasm. One possible interpretation for this is: (Objectives 1, 6)
   A. precursor B ALL
   B. AUL
   C. T-cell ALL
   D. bilineage ALL
4. An adult patient with splenomegaly has an increase in mononuclear cells in the peripheral blood. The bone marrow was filled with a heterogeneous collection of blasts with no granulation. Flow cytometry shows a positive CD20 and CD10 and negative surface immunoglobulin. Which of the following conditions is most likely? (Objective 4)
   A. intermediate pre-B ALL (CALLA)
   B. pro-B ALL
   C. pre-T-cell ALL
   D. cortical T-cell ALL
5. Which of the following represents the most common chromosomal translocation found in ALL? (Objective 4)
   A. t(1;19)(q23;p13.3)/*PBX1-E2A*
   B. t(12;21)(p13;q22)/*ETV6-RUNX1*
   C. t(4;11)(q21;q23)/*MLL-AFF1*
   D. t(9;22)(q34;q11)/*BCR-ABL1*
6. The CD10 antigen found in ALL is also known as the: (Objective 4)
   A. B-cell ALL antigen
   B. T-cell ALL antigen
   C. CALLA antigen
   D. NK-cell ALL antigen
7. The most likely patient presentation for ALL in an adult includes: (Objective 2)
   A. male aged 18–30 with mild anemia and no thrombocytopenia
   B. male older than 60 years with significant anemia and thrombocytopenia
   C. female 10–25 years old with significant bone pain and hemolytic anemia
   D. female 30–50 years old with hemorrhage and bulky nodes
8. The objective of induction therapy includes: (Objective 7)
   A. reduction in the tumor burden
   B. elimination of tumor cells in the central nervous system
   C. replacement with peripheral blood stem cells from another person
   D. prevention of relapse
9. Poor prognostic findings for patients with ALL include: (Objective 8)
   A. young age at time of diagnosis
   B. presence of chromosomal mutations such as *BCR/ABL1*
   C. hyperploidy
   D. presence of *TEL-AML1* gene
10. A large mediastinal mass is found in a 60-year-old male. The bone marrow has 15% lymphoblasts. This is most characteristic of: (Objective 9)
   A. precursor T-cell LBL
   B. ALL—Burkitt's type
   C. precursor T-cell ALL
   D. precursor B-cell ALL

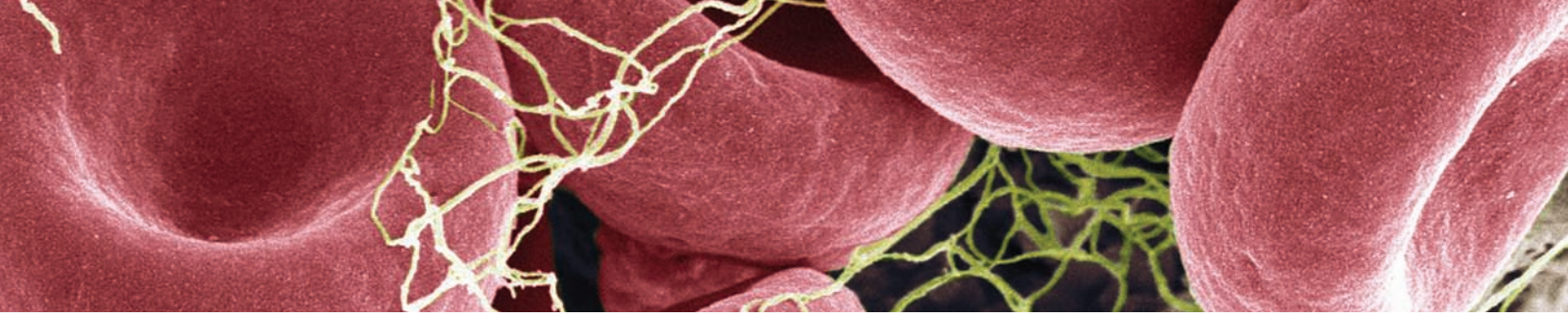
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Pui C. Acute lymphoblastic leukemia. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010;1409–10.
2. Jaffe ES, Harris NL, Stein H et al. Pathology & genetics: tumours of haematopoietic and lymphoid tissues. *World Health Organization Classification of Tumours*. Lyon, France: IARC Press; 2001.
3. Borowitz MJ, Chan JKC. Precursor lymphoid neoplasms. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press, 2008:167–78.
4. Pitman SD, Huang Q. Granular acute lymphoblastic leukemia: a case report and literature review. *Am J Hematol*. 2007;82:834–37.
5. Majumder D, Banerjee D, Chandra S et al. Red cell morphology in leukemia, hypoplastic anemia and myelodysplastic syndrome. *Pathophysiology*. 2006;13:217–25.
6. Kaleem Z, Crawford E, Pathan MH et al. Flow cytometric analysis of acute leukemias: diagnostic utility and critical analysis of data. *Arch Pathol Lab Med*. 2003;127:42–48.
7. Kersey J, Newbit M, Hallgren H et al. Evidence for origin of certain childhood acute lymphoblastic leukemias and lymphomas in thymus-derived lymphocytes. *Cancer*. 1975;36:1348–52.
8. Hurwitz CA, Loken MR, Graham ML et al. Asynchronous antigen expression in B-lineage acute lymphoblastic leukemia. *Blood*. 1988;72:299–307.
9. Gutierrez A, Armstrong SA, Look AT. Pathobiology of acute lymphoblastic leukemia. In: Hoffman R, Furie B, Benz EJ Jr et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier; 2013:935–50.
10. Tyner JW, Jemal AM, Thayer M et al. Targeting surviving and p53 in pediatric acute lymphoblastic leukemia. *Leukemia*. 2012;26:623–32.
11. Kamdar KY, Sandlund JT, Bollard CM. Malignant lymphomas in childhood. In: Hoffman R, Benz EJ Jr, Silberstein LE, eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier. 2013;1255–56.
12. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006;354:166–78.
13. Basso G, Case C, Dell'Orto MC. Diagnosis and genetic subtypes of leukemia combining gene expression and flow cytometry. *Blood Cells Mol Dis*. 2007;39:164–68.
14. Schultz KR, Pullen DJ, Sather HN et al. Risk- and response-based classification of childhood B-precursor acute lymphoblastic leukemia: a combined analysis of prognostic markers from the Pediatric Oncology Group (POG) and Children's Cancer Group (CCG). *Blood*. 2007;109:926–35.
15. Harrison CJ, Haas O, Harbott J et al. Detection of prognostically relevant genetic abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: recommendations from the Biology and Diagnosis Committee of the International Berlin-Frankfürt-Münster study group. *Br J Haematol*. 2010;151:132–42.
16. Ney-Garcia DR, Liehr T, Bhatt S et al. Childhood B-cell progenitor acute lymphoblastic leukemia presenting a three-way t(11;12;21)(q14;p13;q22) with a RUNX1 gene signal on chromosome 11. *Int J Hematol*. 2012;95:112–14.
17. Fuka G, Kauer M, Kofler R et al. The leukemia-specific fusion gene ETV6/RUNX1 perturbs distinct key biological functions primarily by gene repression. *PLoS One*. 2011;6:e26348.
18. Bernt KM, Armstrong SA. Targeting epigenetic programs in MLL-rearranged leukemias. *Hematol Am Soc Hematol Educ Program*. 2011;2011:354–60.
19. Esposito G, Cevenini A, Cuomo A. Protein network study of human AF4 reveals its central role in RNA Pol II-mediated transcription and in phosphorylation-dependent regulatory mechanisms. *Biochem J*. 2011;438:121–31.
20. Xing H, Tang X, Liu T et al. The study of resistance mechanisms and reversal in an imatinib resistant Ph+ acute lymphoblastic leukemia cell line. *Leuk Res*. 2012;36:509–13.
21. Arico M, Valsecchi MG, Camitta B et al. Outcome of treatment in children with Philadelphia chromosome-positive acute lymphoblastic leukemia. *N Engl J Med*. 2000;342(14):998-1006.
22. Najfeld V. Conventional and molecular cytogenetic basis of hematologic malignancies. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier; 2013:728–80.
23. Markovic A, MacKenzie KL, Lock RB. FLT-3: a new focus in the understanding of acute leukemia. *Int J Biochem Cell Biol*. 2005;37:1168–172.
24. Armstrong SA, Look TA. Molecular genetics of acute lymphoblastic leukemia. *J Clin Oncol*. 2005;23:6306–15.
25. Schwab C, Harrison CJ. Acute lymphoblastic leukaemia. *Methods Mol Biol*. 2011;730:99–117.
26. Lewis RE, Cruse JM, Sanders CM et al. The immunophenotype of pre-TALL/LBL revisited. *Exp Mol Pathol*. 2006;81(2):162–65.
27. Kipps TJ, Wang HY. Classification of malignant lymphoid disorders. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill: 2010;1403–408.
28. Sharma VM, Draheim KM, Kelliher MA. The notch1/c-Myc pathway in T cell leukemia. *Cell Cycle*. 2007;6(8):927–30.
29. Borowitz MJ, Bene MC, Harris NL et al. Acute leukemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press, 2008:149–55.
30. Béné MC, Porwit A. Acute leukemias of ambiguous lineage. *Semin Diagn Pathol*. 2012;29:12–28.
31. Scott AA, Head DR, Kopecky KJ et al. HLA-DR$^-$, CD33$^+$, CD56$^+$, CD16$^-$ myeloid/natural killer cell acute leukemia: a previously unrecognized form of acute leukemia potentially misdiagnosed as French-American-British acute myeloid leukemia-M3. *Blood*. 1994; 84:244–55.
32. Garcia Vela JA, Monteserin MC, Delgado I et al. Aberrant immunophenotypes detected by flow cytometry in acute lymphoblastic leukemia. *Leuk Lymphoma*. 2000;36:275–84.



28 Mature Lymphoid Neoplasms

FIONA CRAIG, MD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe the clinical presentation of patients with mature lymphoid neoplasms.
2. Describe how the diagnosis of a lymphoid neoplasm is made.
3. Differentiate among chronic lymphocytic leukemia (CLL), lymphoma, and multiple myeloma based on peripheral blood findings and ancillary studies.
4. Describe the histology of a normal lymph node.
5. Summarize the causes of lymphadenopathy.
6. Contrast the morphology of Hodgkin and non-Hodgkin lymphoma.
7. List and describe the chronic leukemic lymphoproliferative disorders.
8. Recognize and differentiate abnormal and normal lymphocytes on a stained peripheral blood smear and associate their presence with a clinical diagnosis.
9. Describe and apply a multidisciplinary approach to the classification and staging of lymphoid neoplasms.
10. Compare the laboratory and clinical findings of multiple myeloma and lymphoplasmacytic lymphoma.
11. Define *monoclonal gammopathy*.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Describe the use of immunophenotyping and genotyping in detecting clonality and list the characteristic results of the more common mature lymphoid neoplasms.
2. Contrast the features of low-grade and high-grade lymphoma.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

3. Compare and contrast the laboratory features characteristic of the following non-Hodgkin lymphomas:
   a. Small lymphocytic lymphoma
   b. Follicular lymphoma
   c. Mantle cell lymphoma
   d. MALT lymphoma
   e. Lymphoplasmacytic lymphoma
   f. Diffuse large B-cell lymphoma
   g. Burkitt lymphoma
   h. Anaplastic large cell lymphoma
   i. Peripheral T-cell lymphoma
4. Compare and contrast the laboratory features characteristic of Hodgkin lymphoma (HL) subtypes.
5. Compare and contrast the clinical and laboratory features characteristic of the following chronic leukemic lymphoproliferative disorders:
   a. Chronic lymphocytic leukemia (CLL)
   b. B-prolymphocytic leukemia (B-PLL)
   c. Hairy cell leukemia (HCL)
   d. T-prolymphocytic leukemia (T-PLL)
   e. Large granular lymphocytic leukemia (T-LGL)
   f. Sézary syndrome
6. Compare and contrast the laboratory features characteristic of the following plasma cell disorders:
   a. Plasmacytoma
   b. Symptomatic plasma cell myeloma
   c. Monoclonal gammopathy of undetermined significance
7. Recognize and identify the peripheral blood abnormalities associated with CLL, B-PLL, HCL, T-LGL, Sézary syndrome, plasma cell myeloma, and non-Hodgkin lymphoma.
8. Describe the etiology and pathogenesis of lymphoid neoplasms.
9. Differentiate reactive from malignant proliferations of lymphoid cells using clinical and laboratory data.

Key Terms

Anaplastic large cell lymphoma (ALCL)
BCL-2 gene
Bence-Jones proteinuria
Butt cell
Clonality
Dutcher body
Flame cell
Hairy cell
Lacunar cell
Leukemia
Lymphoepithelial lesion
Lymphoma
Monoclonal gammopathy of undetermined significance (MGUS)
Plasma cell neoplasm
Plasmacytoma
Popcorn cell (L&H cell)
Prolymphocyte
Reed-Sternberg (R-S) cell
Richter's transformation
Rouleaux
Sézary cell
Small lymphocytic lymphoma (SLL)
Smudge cell
Stage
Starry-sky appearance
Tingible body macrophages
Tartrate-resistant acid phosphatase (TRAP)
Waldenström macroglobulinemia

Background Basics

The information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the structure and function of lymph nodes. (Chapter 3)
- Outline the classification and summarize the general characteristics of acute leukemia. (Chapter 23)

Level II

- Explain the pathogenesis of neoplasia. (Chapters 2, 23)
- Identify the etiology of acute leukemia. (Chapter 23)

CASE STUDY

We will address this case study throughout the chapter.

Julia, a 56-year-old female, presented with a 4-month history of generalized painless lymphadenopathy and fatigue. A CBC revealed leukocytosis resulting from lymphocytosis.

Consider laboratory features that can help differentiate a reactive lymphocytosis from a neoplastic lymphocytosis.

OVERVIEW

Lymphoid neoplasms can be divided into mature and precursor neoplasms, based on the stage of maturation of the neoplastic cells. The precursor lymphoid neoplasms are discussed in Chapter 27. This chapter focuses on the mature lymphoid neoplasms, and discusses their etiology and pathogenesis, how a diagnosis is established, the classification scheme, and unique features of selected subtypes.

INTRODUCTION

Mature lymphoid neoplasms represent a heterogeneous group of disorders that can be divided into two broad categories based on the distribution of disease: lymphoma and leukemia. **Lymphomas** are malignant neoplasms that present as tumorous masses primarily involving the lymphoid organs, including lymph nodes, tonsils, spleen, thymus, and lymphoid tissue of the gastrointestinal tract. Although most lymphomas are composed of mature lymphoid cells, blastic malignancies do occur (*lymphoblastic lymphoma*). **Leukemia** is a malignant neoplasm that primarily involves the bone marrow and peripheral blood. The chronic leukemic lymphoid neoplasms are composed of mature lymphocytes and usually have an insidious onset and an indolent course. In contrast, lymphoblastic leukemia (also referred to as *acute lymphoblastic leukemia [ALL]*) is composed of blasts belonging to the lymphoid lineage (Chapters 23, 27). Although, in general, these broad categories are useful, distinction between leukemia and lymphoma is not always clear-cut. Tissue involvement can occur in leukemia, and lymphoma can involve the bone marrow and peripheral blood. Indeed, lymphoblastic lymphoma and lymphoblastic leukemia probably represent different clinical manifestations of a single disease entity and are therefore grouped together in the World Health Organization (WHO) classification (lymphoblastic leukemia/lymphoma).[1]

ETIOLOGY AND PATHOGENESIS

The genesis of lymphoid neoplasms is thought to be a multistep process involving acquired genetic, inherited genetic, and environmental factors.

Acquired Genetic Factors

As described for the acute leukemias, acquired alterations of proto-oncogenes and tumor suppressor genes have been associated with the development of mature lymphoid neoplasms. Additional targets for genetic damage are the genes involved in programmed cell death (apoptosis) (e.g., *BCL-2*; Chapter 2). The ***BCL-2* gene** on chromosome 18 is involved in the pathogenesis of follicular lymphoma. Translocation of the *BCL-2* gene to the region of the immunoglobulin heavy chain gene, t(14;18), causes overexpression of the *BCL-2* gene. The resulting increase in BCL-2 protein leads to an inhibition of apoptosis. Decreased cell death results in an accumulation of lymphocytes within the lymph node. Therefore, low-grade follicular lymphoma appears to arise from cell persistence rather than uncontrolled cell proliferation.

Inherited Genetic Factors

Some inherited immunodeficiency syndromes are associated with an increased incidence of malignant lymphoma, including ataxia telangiectasia, Wiskott-Aldrich syndrome, severe combined immunodeficiency (SCID), X-linked lymphoproliferative disorder, and autoimmune lymphoproliferative syndrome (ALPS).

Environmental Factors

Epstein-Barr virus (EBV) is associated with the development of several forms of lymphoid neoplasm including African Burkitt lymphoma, some cases of endemic Burkitt lymphoma, Hodgkin lymphoma (HL), and lymphoma associated with inherited or acquired immunodeficiency. Latent infection with EBV is only one of multiple steps involved in the genesis of these types of lymphoma. EBV infection is acquired orally and is often manifest clinically as infectious mononucleosis. The virus infects B lymphocytes where it remains latent under the immune system's control. The EBV-infected cells can proliferate if the host becomes immunocompromised and/or the B lymphocytes acquire additional genetic abnormalities such as the *C-MYC* translocation.

Another infectious agent associated with the development of non-Hodgkin lymphoma (NHL) is *Helicobacter pylori*. Patients with *H. pylori*-induced inflammation of the stomach have a high incidence of gastric lymphoma of **m**ucosa-**a**ssociated **l**ymphoid **t**issue (MALT) lymphoma type. Chronic *Helicobacter* infection leads to antigen-driven T lymphocyte stimulation and subsequent B lymphocyte activation. The B lymphocytes initially are polyclonal and depend entirely on T lymphocyte stimulation. With time, the B-cell population can proliferate autonomously. If the B lymphocyte proliferation still depends on T lymphocyte stimulation, the lymphoma can regress following removal of the antigenic stimulus with antimicrobial therapy. Lymphoma that is proliferating independent of antigenic stimulation probably requires more drastic therapy including excision and/or chemotherapy.

☑ CHECKPOINT 28-1

How does the *BCL-2* gene rearrangement differ from most other oncogenes?

DIAGNOSIS AND CLASSIFICATION

The diagnosis and classification of mature lymphoid neoplasms involve integration of clinical information with the morphologic appearance and the results of ancillary studies, such as flow cytometry, chromosome analysis, and molecular analysis. For most cases of leukemia, morphologic evaluation is performed on peripheral blood and bone marrow (aspirate and biopsy) samples. A diagnosis of lymphoma is usually rendered from biopsy or a fine-needle aspirate of a mass. In general, normal or reactive proliferations of lymphocytes contain a mixture of cells varying in size, shape, and staining characteristics. The cells present in lymphoid neoplasms are usually more homogeneous than reactive lymphocytes because of the expansion of a single cell type. Less frequently, malignant lymphoid cells can be recognized under the microscope because of an abnormal or bizarre cell appearance.

Often morphology alone is not sufficient to establish a diagnosis of a lymphoid neoplasm and ancillary studies are required. Immunophenotyping, molecular diagnostics, and cytogenetic studies can be used to detect abnormal lymphocytes and/or **clonality** (the presence of identical cells derived from a single progenitor). The lymphoma cells are thought to derive from a single precursor cell (i.e., the cell of origin). The progeny from this cell belong to a clone that shares morphologic, immunophenotypic, and genotypic features. In most circumstances, clonality is synonymous with malignancy. Clonal rearrangement of immunoglobulin or T-cell receptor

genes or the presence of an abnormal translocation can be used to identify neoplastic lymphocytes (Chapter 42). The presence of characteristic translocations can also assist in the diagnosis of a lymphoid neoplasm as well as determining the subtype, as in the *BCL-2* gene rearrangement in follicular lymphoma (Chapters 41 and 42). Flow cytometric studies can be used to infer clonality by demonstrating expression of only one of the immunoglobulin light chains (κ or λ) on B cells or the presence of a population of cells with an abnormal phenotype such as CD5− T lymphocytes (Chapter 40). In addition, the phenotype of the neoplastic cells often assists in classifying a lymphoid neoplasm, determining prognosis, and detecting residual disease following treatment.

After a diagnosis of a lymphoid neoplasm has been established, classification is performed to guide therapy and provide additional prognostic information. The World Health Organization (WHO) scheme currently classifies mature lymphoid neoplasms into distinct disease entities that have similar morphologic, phenotypic, and genotypic features[1] (Table 28-1 ★). Some of these disease entities have a long indolent course and are often referred to as being low-grade (e.g., small lymphocytic lymphoma). In contrast, some subtypes are clinically aggressive and kill the patient rapidly without treatment and are referred to as *aggressive* or *high grade* (e.g., Burkitt lymphoma). Current therapeutic regimens are actually more effective at treating high-grade than low-grade lymphoid neoplasms, and many patients treated for high-grade lymphoma are cured of their disease. Often patients with low-grade lymphoid neoplasms are treated for symptomatic relief rather than the intent to cure. In general, histologic sections from lower-grade lymphoma more often demonstrate a nodular growth pattern, smaller cells, lower mitotic activity, and an absence of apoptosis. Higher-grade lymphoma usually has a diffuse growth pattern, is often composed of larger cells, and displays more numerous mitoses and apoptotic bodies.

In addition to the prognostic information provided by identifying the disease entities defined in the WHO classification, some can be divided into further prognostic groups. For instance, the entity follicular lymphoma is divided into three grades defined by the number of large cells (centroblasts). Many prognostic markers have been used to identify cases of chronic lymphocytic leukemia that will have a more aggressive course than expected, including expression of the proteins CD38 and ZAP-70, presence of cytogenetic abnormalities, and degree of mutation of the immunoglobulin heavy chain variable region. Diffuse large B-cell lymphoma is divided into germinal and nongerminal center types with a significantly better clinical outcome for patients with the germinal center subtype. However, the prognosis of a patient with a lymphoid neoplasm is not only related to the pathologic findings but also clinical parameters, such as the extent and

★ **TABLE 28-1** WHO Classification of Selected Mature Lymphoid Neoplasms

	Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma
Mature B-Cell Neoplasms	B-cell prolymphocytic leukemia
	Hairy cell leukemia
	Follicular lymphoma
	Mantle cell lymphoma
	Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
	Splenic marginal zone lymphoma
	Nodal marginal zone lymphoma
	Lymphoplasmacytic lymphoma
	Diffuse large B-cell lymphoma (DLBCL), not otherwise specified
	Primary mediastinal (thymic) B-cell lymphoma
	Burkitt lymphoma
	Plasma cell neoplasms: plasma cell myeloma, monoclonal gammopathy of undetermined significance, plasmacytoma, monoclonal immunoglobulin deposition diseases
Mature T- and NK-cell Neoplasms	T-cell prolymphocytic leukemia
	T-cell large granular lymphocytic leukemia
	Sézary syndrome
	Adult T-cell leukemia/lymphoma
	Extranodal NK/T-cell lymphoma, nasal type
	Enteropathy-associated T-cell lymphoma
	Hepatosplenic T-cell lymphoma
	Subcutaneous panniculitis-like T-cell lymphoma
	Angioimmunoblastic T-cell lymphoma
	Anaplastic large cell lymphoma (ALCL), ALK positive
	Anaplastic large cell lymphoma (ALCL), ALK negative
	Peripheral T-cell lymphoma, not otherwise specified
Hodgkin lymphoma	Nodular lymphocyte predominant Hodgkin lymphoma
	Classical Hodgkin lymphoma: nodular sclerosis, mixed cellularity, lymphocyte rich, and lymphocyte depleted

★ **TABLE 28-2** Ann Arbor Staging System for Malignant Lymphoma

I	Single lymph node region or single extralymphatic site (I_E)
II	Two or more lymph node regions on same side of diaphragm or with involvement of limited contiguous extralymphatic site (II_E)
III	Lymph node regions on both sides of diaphragm, which can include spleen (III_S) and/or limited contiguous extralymphatic site (III_E)
IV	Multiple or disseminated foci of involvement of one or more extralymphatic organs or tissues with or without lymphatic involvement

distribution of disease (**stage**). Patients with widespread lymphoma usually have a worse prognosis. Determining the stage of disease usually involves radiologic studies, peripheral blood examination, and bone marrow aspiration and biopsy. The Ann Arbor scheme is often used to stage malignant lymphoma (Table 28-2 ★). Bone marrow involvement indicates disseminated disease, Stage IV.

The remainder of this chapter describes examples of mature lymphoid neoplasms that have been selected either because they are more common or illustrate important concepts. A more complete listing and description of subtypes of mature lymphoid neoplasms is available.[1]

☑ CHECKPOINT 28-2

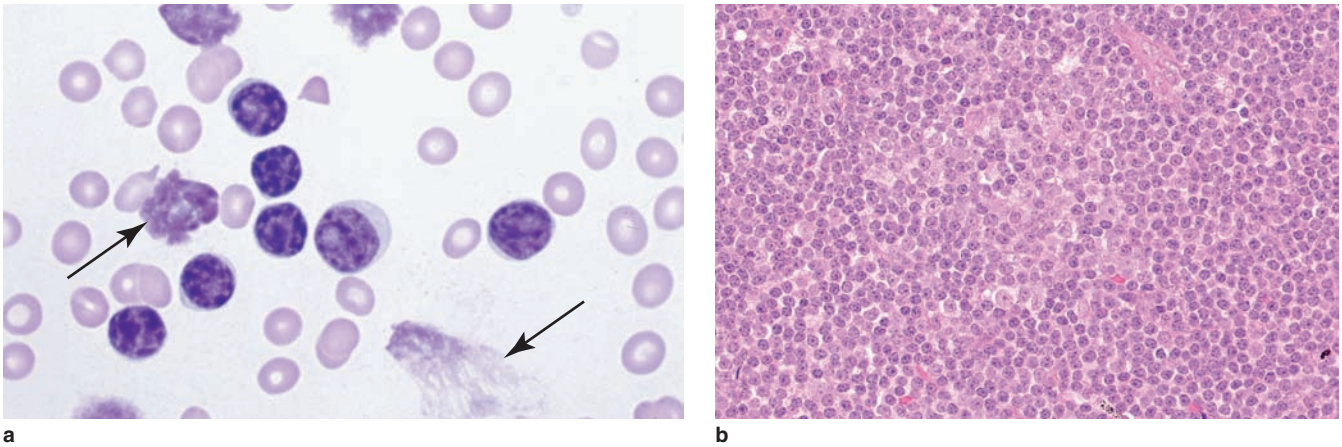
How does staging differ from grading in characterizing the lymphoid neoplasms?

MATURE B-CELL NEOPLASMS

Chronic Lymphocytic Leukemia/ Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) seem to represent different clinical manifestations of one disease entity (CLL/SLL). Patients with CLL present with peripheral blood lymphocytosis but often develop lymph node involvement. In contrast, patients with SLL present with lymphadenopathy but often develop peripheral blood and bone marrow disease. CLL/SLL can be detected in an asymptomatic patient as the result of finding lymphocytosis on a CBC performed for another reason. When present, symptoms are often related to anemia, thrombocytopenia, and neutropenia. These cytopenias can arise from a variety of causes including replacement of the bone marrow hematopoietic cells by neoplastic lymphocytes, hypersplenism, poor nutritional status, and immune-mediated cell destruction (Chapter 19).

A diagnosis of CLL requires a sustained absolute lymphocytosis $>5 \times 10^9$/L with characteristic morphologic and phenotypic findings. The lymphocytes are small and appear mature with scant cytoplasm. The nuclei are usually round, and the chromatin is regularly clumped (block-type chromatin). Nucleoli are inconspicuous (Figure 28-1a ■). A few large **prolymphocytes** are usually present but represent <10% of all lymphocytes (Figure 28-1a). Prolymphocytes have abundant pale-staining cytoplasm and a large central prominent nucleolus. The neoplastic cells in CLL appear to be more fragile than normal lymphocytes and often burst open during smear preparation, producing **smudge cells** (Figure 28-1a). However, smudge cells also can be found in reactive lymphocytosis and in other neoplasms; therefore, their presence should not be used to diagnose CLL. In fact, smudge cells can make diagnosing CLL more difficult by preventing visualization of the lymphocytes. The number of smudge cells can be reduced by mixing a drop of albumin with a drop of blood prior to making the smear.



a b

■ **FIGURE 28-1** **(a)** Chronic lymphocytic leukemia. Small round lymphocytes with clumped chromatin, a larger prolymphocyte with a prominent nucleolus, and numerous smudge cells (arrows) (peripheral blood, Wright stain, 1000× magnification). **(b)** Small lymphocytic lymphoma. The periphery of the image displays many small lymphocytes with round nuclei and clumped chromatin. The center of the image contains a vague nodule containing pale-staining larger cells (proliferation center) (lymph node biopsy, H&E stain, 50× magnification).

The differential diagnosis of CLL includes reactive lymphocytosis and the other chronic leukemia lymphoproliferative disorders. Therefore, although a provisional diagnosis usually can be made following smear examination, immunophenotyping is often used to establish a definitive diagnosis. CLL is characterized by aberrant expression of the T lymphocyte antigen CD5. The CLL cells usually have weak surface expression of CD20 and monoclonal immunoglobulin. Expression of CD23 and CD200 and lack of FMC-7 positivity distinguishes CLL from leukemic mantle cell lymphoma (MCL). CLL and MCL have many of the same features that can make differential diagnosis difficult.[2] Flow cytometric studies occasionally detect a small population of cells with a phenotype characteristic of CLL but representing less than the required absolute count of 5×10^9/L. If there is no clinical evidence of an overt lymphoid neoplasm, the significance of this finding is uncertain and is referred to as *monoclonal B lymphocytosis (MBL)*.

A lymph node biopsy performed in CLL/SLL reveals an essentially diffuse infiltrate of small mature lymphocytes. The small lymphocytes have dense, regularly clumped chromatin and lack nucleoli (Figure 28-1b). At low power, a vague nodularity is often present as a result of aggregates of paler large cells (proliferation centers) (Figure 28-1b). Proliferation centers contain larger cells with abundant pale-staining cytoplasm and prominent eosinophilic nucleoli (including prolymphocytes).

CLL/SLL had been considered a low-grade disease with a uniformly indolent course that could not be cured and was treated only for symptom relief. More recently, it has been recognized that CLL/SLL is actually a rather heterogeneous disorder that includes a subset of patients that requires earlier treatment and has a shorter overall survival than others in this group. Several prognostic markers have been associated with this difference in clinical behavior and are often evaluated at diagnosis in an attempt to predict outcome. These prognostic markers include the proteins CD38 and ZAP-70 and the mutational status of the immunoglobulin (Ig) heavy chain gene variable region (V_H). During normal B-cell development, rearranged immunoglobulin variable gene segments undergo somatic hypermutation. CLL cases can be divided into two groups, those with "mutated" V_H genes and those with "unmutated" V_H genes. CLL with a more aggressive behavior is associated with higher levels of expression of CD38 and ZAP-70 as determined by flow cytometry and an unmutated Ig V_H gene.

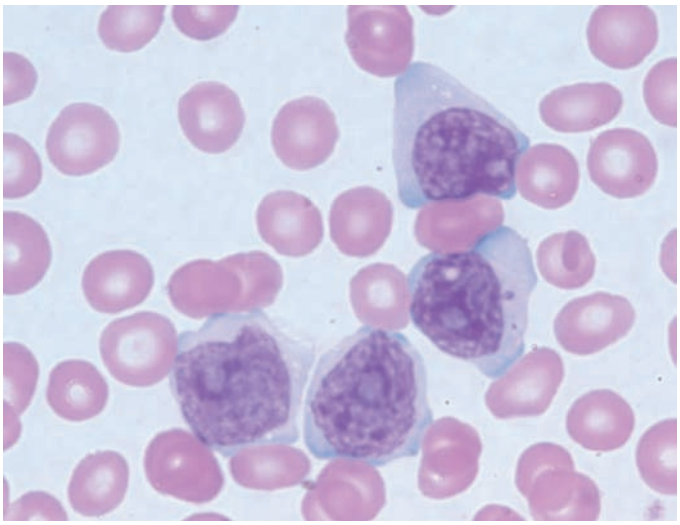
Cytogenetic abnormalities were initially difficult to detect in CLL/SLL because the leukemic cells were not easy to induce to proliferate in culture. However, with improved cytogenetic methods such as fluorescence in situ hybridization (FISH), approximately half of all CLL/SLL patients are found to have clonal chromosomal abnormalities. The most common chromosomal abnormality, del 13q14-23.1, is associated with a relatively good prognosis. The following chromosomal abnormalities, listed in order of decreasing frequency, are associated with a worse prognosis:

- Trisomy 12
- Del 11q22.3-23.1
- Del 6q21-23
- Deletions at 17p13.1 (*p53* aberrations)
- 14q abnormalities and complex chromosomal abnormalities

Less than 10% of patients with CLL/SLL develop transformation to a B-cell lymphoid neoplasm with a worse prognosis. Over time, the number of prolymphocytes can increase, often in association with acquired genetic abnormalities, although progression to prolymphocytic leukemia is unusual. **Richter's transformation** is the transition to an aggressive large B-cell lymphoma, which occurs in approximately 2–8% of patients with CLL/SLL. The Rai and Binet prognostic staging systems, which are based on physical examination and blood counts, can be used to assess the need for therapy and the specific type of therapy.[3] Typically, patients with early stage CLL do not receive therapy but are monitored for disease progression. Standard therapy for those who are symptomatic or have advanced disease is chemotherapy with fludarabine, cyclophosphamide, and rituximab (anti-CD20).[3] New small-molecule inhibitors (e.g., ibrutinib) that work inside the cell to block activity of enzymes responsible for cell signaling may change the standard treatment of B-cell malignancies.

B-Cell Prolymphocytic Leukemia

B-cell prolymphocytic leukemia (B-PLL) is an aggressive, rare, leukemic disorder that often does not respond to treatment. Although most cases arise de novo, B-PLL rarely can develop from CLL (refer back to "Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma"). Unlike CLL, patients with B-PLL usually have prominent splenomegaly but minimal lymphadenopathy. The CBC in patients with B-PLL reveals marked absolute lymphocytosis, often $>300 \times 10^9$/L, anemia, and thrombocytopenia. The neoplastic cells have a characteristic appearance: large with moderate amounts of pale basophilic cytoplasm, moderately condensed chromatin, and a single prominent nucleolus (Figure 28-2 ■). Prolymphocytes represent <10% of the lymphocytes seen in CLL and >55% of the lymphocytes present in PLL. Cases with 11–55% prolymphocytes have an unpredictable course. The phenotype of B-PLL often differs from that of CLL in demonstrating more variable expression of CD5, stronger intensity expression of surface immunoglobulin and CD20, positivity with



■ **FIGURE 28-2** Prolymphocytic leukemia. Numerous large lymphoid cells with prominent nucleoli (prolymphocytes) (peripheral blood, Wright stain, 1000× magnification).

FMC-7, and absence of CD23. B-PLL is not associated with a characteristic chromosome abnormality, but the short arm of chromosome 17 is often deleted, del(17p), resulting in inactivation of the *p53* gene (a tumor suppressor gene). B-PLL does not respond well to chemotherapy and has a poor prognosis.

Hairy Cell Leukemia

Hairy cell leukemia (HCL) (Figure 28-3 ■) is an uncommon B-cell neoplasm presenting in middle age. Males have a significantly higher incidence (male-to-female ratio = 7:1). At presentation, patients usually have massive splenomegaly but lack lymphadenopathy. Extensive bone marrow involvement often exists; therefore, patients with HCL usually present with pancytopenia. The white blood cell (WBC) count is low because of both neutropenia and monocytopenia; patients thus have an increased susceptibility to infection, especially with mycobacterial organisms. Although neoplastic cells (**hairy cells**) are usually present on a peripheral smear, they are usually too few to elevate the WBC count. The hairy cells have a characteristic abnormal appearance with abundant pale-staining cytoplasm, circumferential cytoplasmic projections ("hairs"), usually oval or reniform nuclei, and relatively fine chromatin (Figure 28-3a). Flow cytometric immunophenotyping is used to establish a diagnosis of HCL. HCL cells are mature B lymphocytes that are positive for CD19, CD20 (strong intensity), CD22, CD25, CD103, and CD11c. There is usually strong-intensity monoclonal surface immunoglobulin present. In addition, the cells of HCL show acid phosphatase staining after tartrate incubation (**tartrate-resistant acid phosphatase [TRAP]**).

Bone marrow involvement is often diffuse, and the neoplastic cells are usually surrounded by fibrosis, preventing their aspiration from the bone marrow (resulting in a "dry tap"). Bone marrow biopsy sections reveal a monotonous infiltrate of abnormal lymphocytes with small nuclei and abundant pale-staining cytoplasm ("fried egg" appearance) (Figure 28-3b). Splenectomy specimens reveal marked expansion of the red pulp because of an infiltrate of abnormal cells with the fried egg appearance. Lakes of erythrocytes are often formed between the tumor cells (pseudosinuses). Immunohistochemical stains for the B-cell antigen CD20 can be used to highlight the infiltrate. Other features characteristic of HCL include Annexin A1 and CD123 staining and *BRAF* V600E mutation.[4–6] HCL is an indolent disease. Long-lasting complete remissions are often obtained with the chemotherapy agent 2-chlorodeoxyadenosine (2-CDA/cladribine).

☑ CHECKPOINT 28-3

Chronic lymphoid malignancies compose a heterogeneous group. What characteristics allow these malignancies to be grouped together?

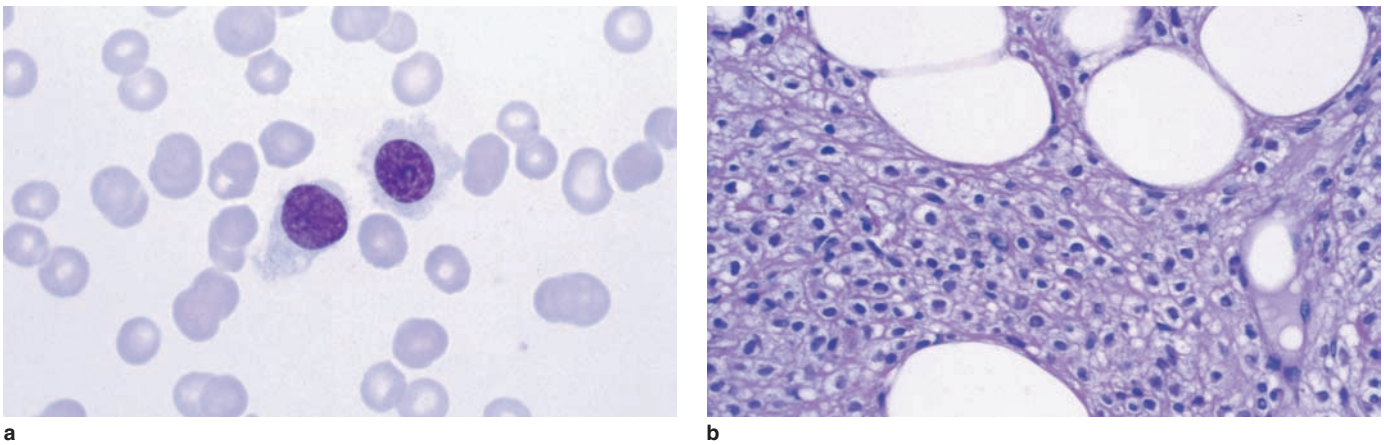
CASE STUDY (continued from page 536)

Julia's CBC revealed a WBC count of 20 × 10^9/L with 60% lymphocytes. Examination of the peripheral blood revealed mature lymphocytes with scant cytoplasm, clumped chromatin, and irregular nuclear outlines.

1. What is the differential diagnosis?
2. What studies could be performed to establish the diagnosis?

Follicular Lymphoma

Follicular lymphoma (FL) (grade 1; Figure 28-4 ■) is one of the most common types of mature B-cell lymphoid neoplasm within the United States, second only to diffuse large B-cell lymphoma. Patients usually present with generalized painless lymphadenopathy, most have advanced stage disease (Figure 28-4a) with bone marrow involvement (Figure 28-4a), and a few have peripheral blood involvement (Figure 28-4b). Involvement of other extranodal sites is unusual.



a b

■ **FIGURE 28-3** Hairy cell leukemia. **(a)** Two abnormal lymphocytes with abundant pale-staining cytoplasm with hairlike projections and relatively finely distributed chromatin (peripheral blood, Wright stain, 1000× magnification). **(b)** Replacement of hematopoietic precursors by abnormal small lymphocytes with abundant clear cytoplasm ("fried-egg" appearance) (bone marrow, H&E stain, 1000× magnification).

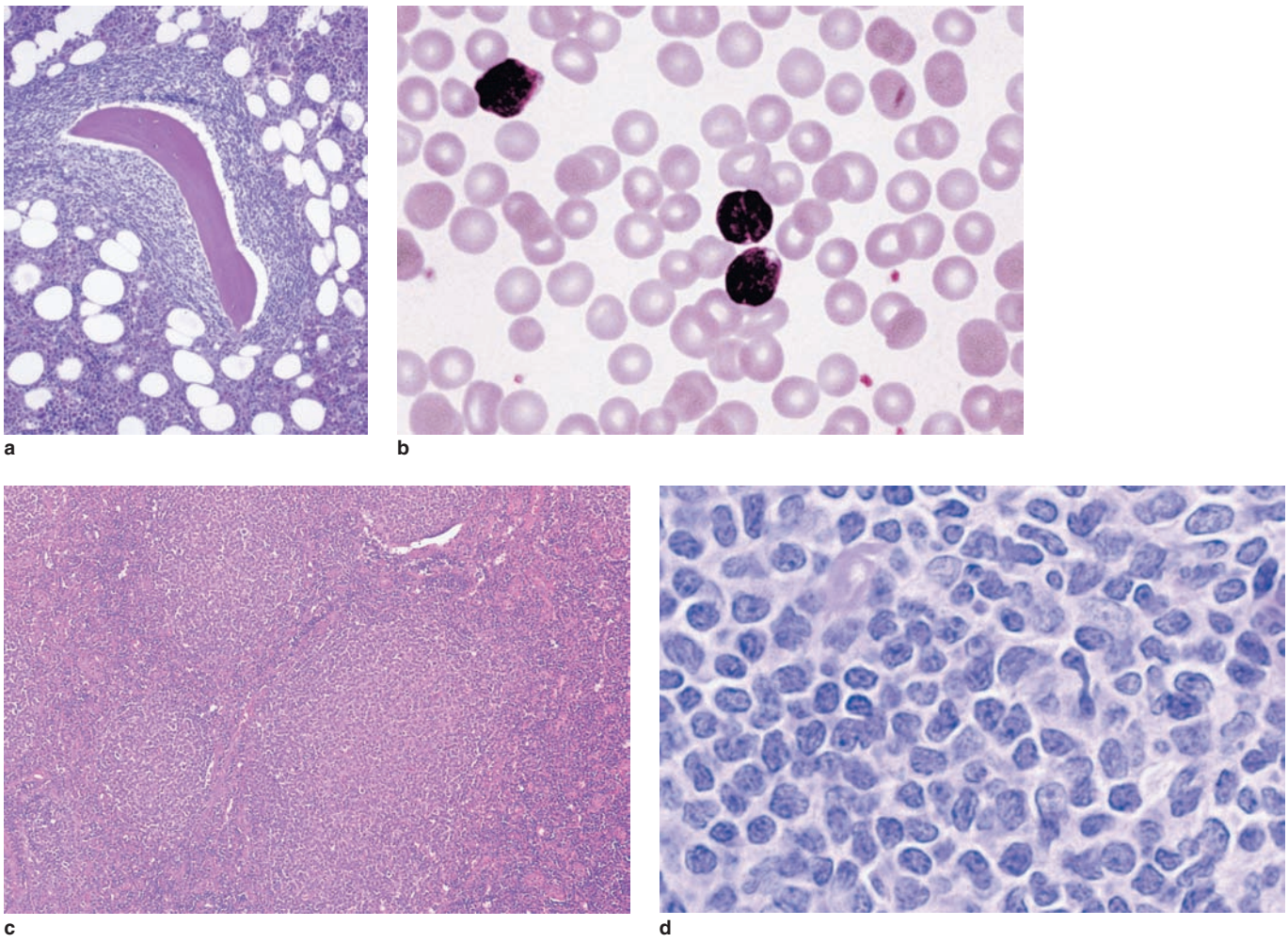


FIGURE 28-4 Follicular lymphoma, grade 1. **(a)** Bone marrow involvement by low grade follicular lymphoma displaying a characteristic paratrabecular growth pattern (bone marrow biopsy, H&E stain, 20× magnification). **(b)** Circulating lymphoma cells with irregular nuclear outlines (peripheral blood, Wright stain, 1000× magnification). **(c)** Loss of the normal lymph node architecture. The abnormal infiltrate forms numerous poorly defined nodules (follicles) (lymph node biopsy, H&E stain, 20× magnification). **(d)** Neoplastic follicle composed of a relatively homogeneous population of small lymphoid cells containing angulated, twisted nuclei (lymph node biopsy, H&E stain, 200× magnification).

Lymph node biopsy of FL reveals an infiltrate of lymphoid cells forming poorly circumscribed nodules that resemble follicular germinal centers (Figure 28-4c). In addition, the lymphoma cells grow in a diffuse pattern in some areas. Neoplastic follicles differ from reactive follicles in that neoplastic follicles lack evidence of lymphocyte apoptosis. This is manifest on histologic sections as a lack of macrophages engulfing fragments of dead cells (**tingible body macrophages**), which are usually found in areas of extensive apoptosis (reactive germinal centers and high-grade lymphoma). The neoplastic infiltrate always contains a mixed population of small cleaved (centrocytes) and large cells (centroblasts) but is often more homogeneous than a normal germinal center (Figure 28-4d). The proportion of large cells varies and is used to separate FL into three grades (1, 2, or 3) (Table 28-3 ★). The presence of more large cells is often associated with a more aggressive clinical course. FL cells in the peripheral blood and bone marrow usually have very irregular nuclear outlines and a deep indentation (cleft) of the nuclear membrane ("buttock" or **"butt" cells**) (Figure 28-4b).

Conventional histologic evaluation is usually insufficient to establish a diagnosis of FL. Immunophenotyping can be used to confirm the presence of lymphoma (monoclonal lymphocytes) and confirm the subtype of lymphoma (CD10+). Most FL cases arise because of a chromosome translocation t(14;18) involving the *BCL-2* gene that leads to overexpression of BCL-2 protein in lymphocytes. BCL-2 protein inhibits individual cell death (apoptosis), allowing follicle center cells to accumulate and produce lymphadenopathy.

★ **TABLE 28-3** Grading of Follicular Lymphoma

Grade	Definition
1	0–5 centroblasts per defined high-power field
2	6–15 centroblasts per defined high-power field
3	>15 centroblasts per defined high-power field

Most patients with FL have an indolent disease with a median survival of 7–9 years. Patients with grade 3 FL are sometimes cured. However, the lower-grade FLs (grades 1 and 2) are incurable with current therapies and are therefore treated for relief of symptoms. Low-grade FL can progress to a diffuse large cell lymphoma with a median survival of <1 year.

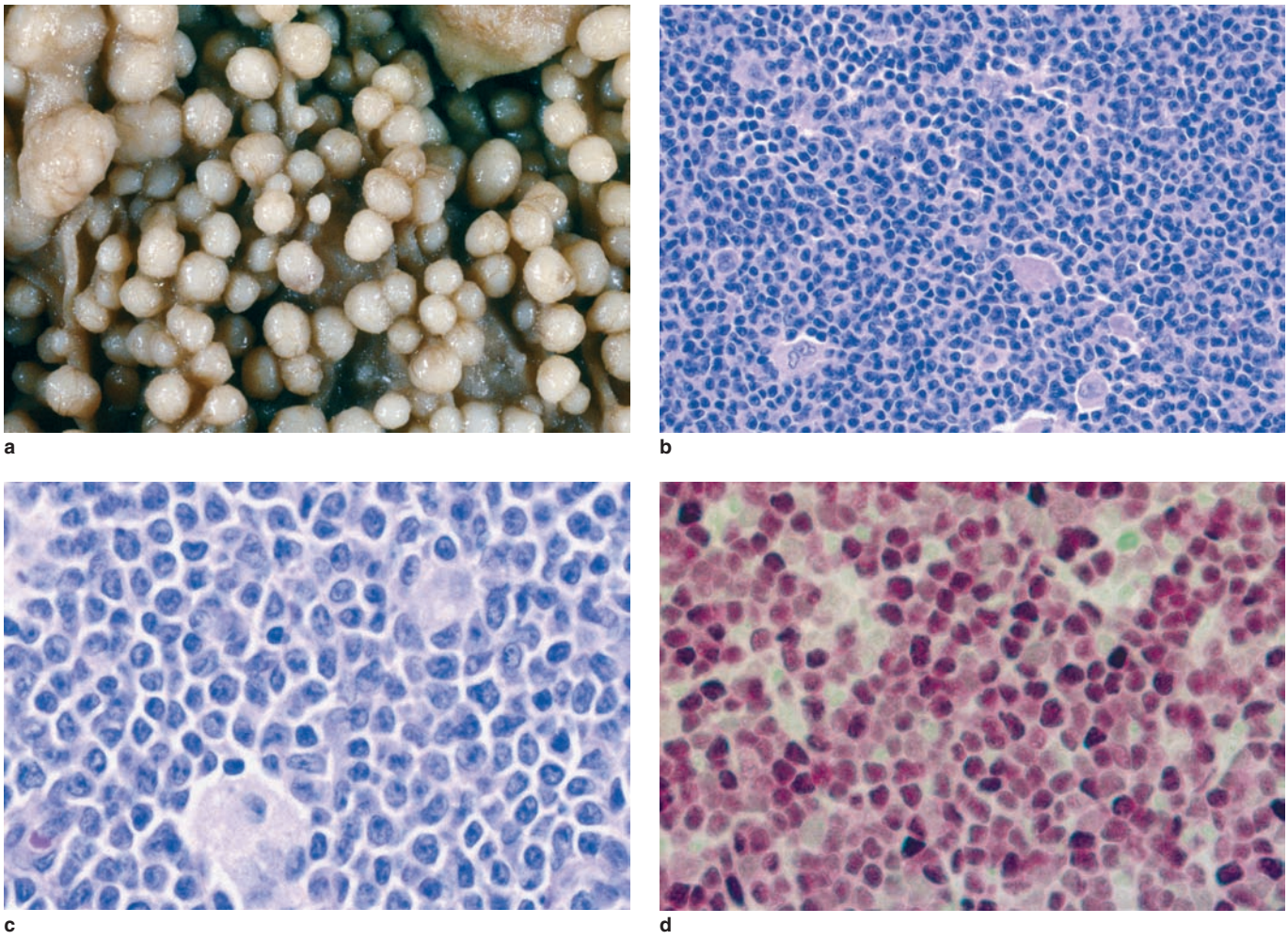
CASE STUDY *(continued from page 541)*

Julia's cervical lymph node biopsy reveals effacement of the normal architecture by a lymphoid infiltrate with a nodular growth pattern. The nodules contain a relatively homogeneous population of small lymphocytes and a few admixed large cells. The infiltrating lymphoid cells have irregular nuclear outlines. Tingible body macrophages are lacking, and very few mitotic figures are present.

3. What is the cause of the lymphadenopathy?
4. Is this process low grade or high grade?

Mantle Cell Lymphoma (MCL)

Patients who have mantle cell lymphoma (MCL) (Figure 28-5 ■) usually present with disseminated disease involving multiple lymph node groups, bone marrow, peripheral blood, spleen, liver, and gastrointestinal tract. Gastrointestinal tract involvement can present as multiple polyps involving the small bowel (lymphomatous polyposis) (Figure 28-5a). On histologic sections, MCL can demonstrate either a diffuse or vaguely nodular growth pattern (Figure 28-5b). MCL with a nodular growth pattern can be difficult to distinguish from FL by morphologic evaluation alone. The neoplastic infiltrate occasionally surrounds a reactive germinal center (mantle zone pattern). The neoplastic cells are usually small to intermediate in size with round to slightly irregular nuclear outlines (Figure 28-5c). However, there are morphologic variants of MCL with cells resembling lymphoblasts (blastoid MCL) or large cells (pleomorphic MCL). Establishing an MCL diagnosis usually requires the use of ancillary studies. The following immunophenotype is characteristic of MCL: CD19+, CD5+, CD23−, FMC-7+, and sIg + (strong intensity),



a **b** **c** **d**

■ **FIGURE 28-5** Mantle cell lymphoma. **(a)** Gross photograph of the large intestine in a patient with lymphomatous polyposis of gastrointestinal tract. **(b)** Abnormal lymphoid infiltrate in a lymph node displaying a vaguely nodular growth pattern (lymph node biopsy, H&E stain, 20× magnification). **(c)** Uniform infiltrate of small lymphoid cells with slightly irregular nuclear outlines (lymph node biopsy, H&E stain, 200× magnification). **(d)** Cyclin D1 nuclear overexpression (lymph node biopsy, immunohistochemistry stain, 100× magnification).

and almost all are positive for cyclin D1 by immunohistochemistry (Figure 28-5d). Cyclin D1 is involved in regulating progression of cells from the G_1 to S phase of the cell cycle (Chapter 2). Overexpression of cyclin D1 in MCL is usually the result of a chromosome translocation, t(11;14), which involves the *BCL-1* gene. The *BCL-1* translocation is thought to lead to neoplastic transformation through loss of cell-cycle control. This translocation can be demonstrated by FISH studies in the vast majority of cases of MCL.

MCL is a relatively aggressive lymphoma with a poor response to current therapies. However, recent findings regarding the pathogenesis of the disease may help in the development of new therapeutic approaches.[7] Currently, the overall median survival is approximately 3–4 years. Therefore, distinction of MCL from the other small lymphoid B-cell neoplasms (follicular lymphoma, SLL, and MALT lymphoma [see "Extranodal Marginal Zone Lymphoma of Mucosa Associated Lymphoid Tissue"]) is important.

Extranodal Marginal Zone Lymphoma of Mucosa Associated Lymphoid Tissue

Patients with extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT) usually present with localized extranodal disease (e.g., involving the stomach, salivary gland, lacrimal gland, thyroid, lung). A preceding chronic inflammatory disorder such as chronic gastritis from *Helicobacter pylori* infection or autoimmune disease (Sjögren's syndrome or Hashimoto's thyroiditis) often occurs.

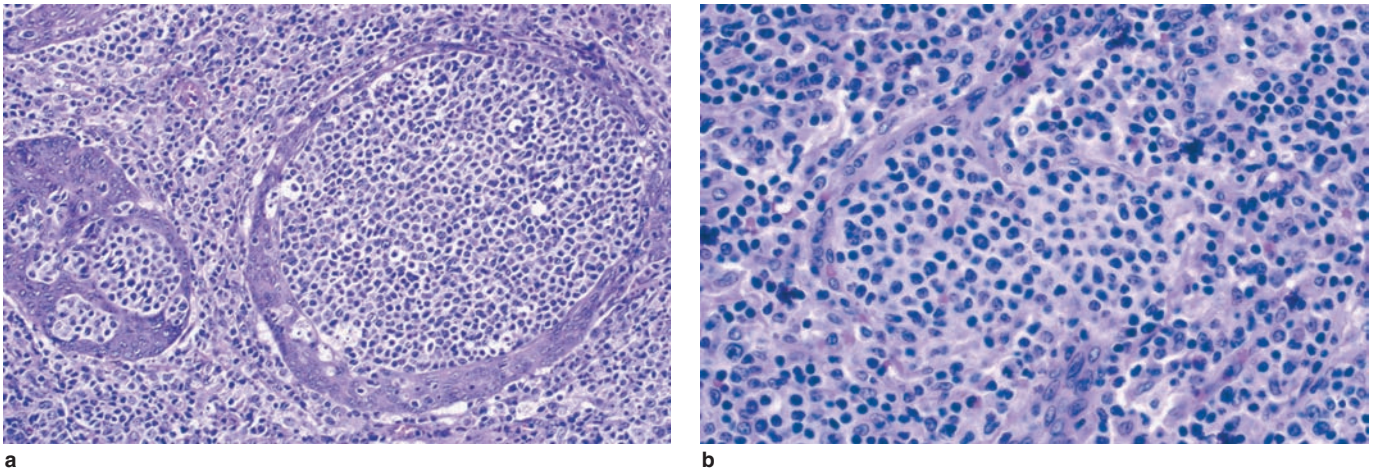
A biopsy of MALT lymphoma (Figure 28-6a ■) reveals an infiltrate of small to intermediate size lymphocytes intimately associated with epithelial cells (e.g., gastric mucosa, salivary gland ducts). Epithelial structures infiltrated by neoplastic lymphocytes are referred to as **lymphoepithelial lesions** (Figure 28-6a). MALT lymphoma is composed predominantly of small lymphocytes with round or slightly cleaved nuclei that often have abundant pale-staining cytoplasm and are referred to as *monocytoid B cells* because of their resemblance to monocytes (Figure 28-6b). MALT lymphoma can contain admixed neoplastic plasma cells. In addition to the neoplastic cells, the infiltrate often contains benign germinal centers. Infiltration of benign germinal centers by neoplastic cells is referred to as *follicular colonization*.

The differential diagnosis includes other lymphomas composed of small lymphocytes, including SLL, MCL, and FL. Ancillary studies can assist in obtaining the correct diagnosis. Restriction of immunoglobulin light chains can be demonstrated by immunophenotyping (surface immunoglobulin on lymphocytes) or paraffin section immunohistochemistry (cytoplasmic immunoglobulin in cells demonstrating plasma cell differentiation). In contrast to FL and small lymphocytic lymphoma, MALT lymphoma is usually negative for CD10 and CD5.

MALT lymphoma is an indolent disease previously considered to be a reactive condition mimicking lymphoma (pseudolymphoma) but is now considered malignant because, like other NHLs, it is composed of monoclonal lymphocytes and has the potential to transform to higher-grade, large B-cell lymphoma. Although, patients with *H. pylori*–associated gastric MALT lymphoma are often cured with antimicrobial therapy, the polymerase chain reaction (PCR) assay for a monoclonal B-cell population can remain positive after complete remission.[8] MALT lymphoma that is unresponsive to antimicrobial therapy or that occurs at other sites is often treated with local excision or radiation therapy.

Lymphoplasmacytic Lymphoma

Lymphoplasmacytic lymphoma (LPL) is a neoplasm that primarily involves the bone marrow and sometimes the spleen and lymph nodes and is composed of a mixture of neoplastic small lymphocytes and



a b

■ **FIGURE 28-6** Extranodal marginal zone lymphoma of mucosa-associated slymphoid tissue (MALT lymphoma). **(a)** Parotid salivary gland involved by MALT lymphoma. Two lymphoepithelial lesions are present demonstrating infiltration of ducts by neoplastic lymphoid cells (parotid gland biopsy, H&E stain, 50× magnification). **(b)** Lymphoid infiltrate of MALT lymphoma. Many of the neoplastic cells have abundant pale-staining cytoplasm (i.e., a "monocytoid" appearance) (parotid gland biopsy, H&E stain, 100× magnification).

plasma cells. LPL must be distinguished from small B-cell lymphoid neoplasms, other types of lymphoma that can have plasmacytic differentiation, such as MALT lymphoma, and neoplasms composed entirely of plasma cells (plasma cell neoplasms). Unlike those with plasma cell neoplasm multiple myeloma, patients with LPL lack lytic bone lesions. The neoplastic plasma cells in LPL often secrete monotypic immunoglobulin, leading to a paraprotein, often of IgM type. High plasma levels of the large pentamer IgM result in hyperviscosity that can lead to poor circulation in small blood vessels, visual impairment, headache, dizziness, and deafness. **Waldenström macroglobulinemia** is the combination of LPL with bone marrow involvement and an IgM monoclonal paraprotein.

Histologic sections usually demonstrate a diffuse infiltrate composed of lymphocytes, plasma cells, and lymphoid cells that demonstrate some evidence of differentiation toward plasma cells (plasmacytoid lymphocytes). Immunohistochemistry can be performed to demonstrate the presence of cytoplasmic monoclonal immunoglobulin light chain (κ or λ) and IgM heavy chain.

Treatment of symptomatic patients includes rituximab, which can be used in combination with chemotherapy. Autologous bone marrow transplant is a recent treatment option. Plasmapheresis to treat hyperviscosity is used to reduce paraprotein levels. The median survival for patients with LPL is typically 5–10 years.

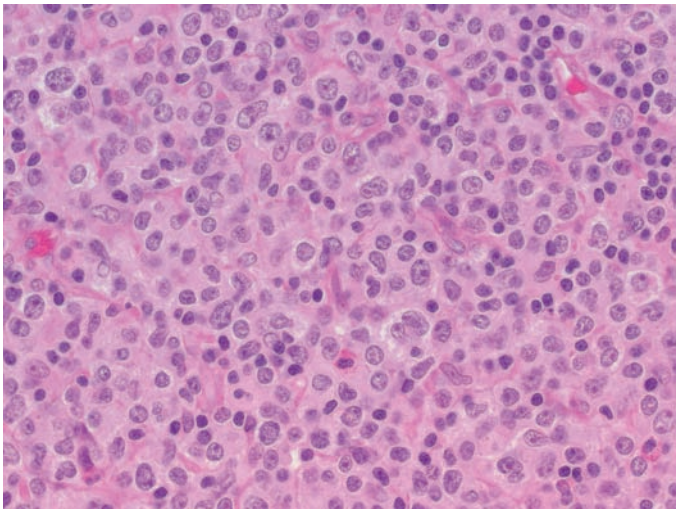
Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of tumors composed of large B-lymphoid cells (Figure 28-7 ■). Some DLBCLs develop as a result of transformation from a lower-grade lymphoma (e.g., follicular lymphoma or small lymphocytic lymphoma), whereas others arise de novo. The possibility of the pleomorphic variant of mantle cell lymphoma should be excluded using lack of staining for cyclin D1 in DLBCL. The immunophenotype of DLBCL is quite variable and has been associated with the clinical course. Diffuse large B-cell lymphoma with a phenotype similar to follicular lymphoma (germinal center phenotype) often has a better prognosis than does diffuse large B-cell lymphoma with a nongerminal center phenotype. With multiagent chemotherapy and anti-CD20 monoclonal antibody therapy (rituximab), the long-term remission rate of DLBCL is 50–60%.

CASE STUDY *(continued from page 543)*

The previous biopsy had established a diagnosis of low-grade NHL, follicular type. Julia received multiagent chemotherapy for symptomatic relief. Two years following the diagnosis, she returned with rapidly expanding lymph nodes in her neck. Repeat biopsy revealed effacement of the lymph node architecture by a diffuse infiltrate of large B cells.

5. What is the diagnosis?
6. What is the relationship of this disease to the previous diagnosis?



■ **FIGURE 28-7** Diffuse large B-cell lymphoma. Abnormal infiltrate composed of large lymphoid cells with pale-staining, vesicular chromatin, irregular nuclear outlines, and basophilic nucleoli. Several mitotic figures are present (lymph node biopsy, H&E stain, 200× magnification).

Burkitt Lymphoma

Burkitt lymphoma is a high-grade NHL with a high incidence in Africa (endemic subtype). It represents approximately one-third of all pediatric lymphomas occurring outside Africa (sporadic Burkitt lymphoma). Many adult cases occur in immunocompromised individuals such as those infected with the HIV virus. Burkitt lymphoma often involves extranodal sites. Endemic Burkitt lymphoma has a predilection for involvement of the facial bones and jaw; sporadic Burkitt lymphoma often presents with disease involving the intestine, ovaries, or kidney. EBV is thought to play a role in the pathogenesis of Burkitt lymphoma. DNA of EBV is present in most cases of endemic Burkitt lymphoma and approximately one-third of the HIV-associated tumors. EBV is found less frequently in the sporadic form.

A biopsy of Burkitt lymphoma usually reveals a diffuse infiltrate of neoplastic cells demonstrating a **"starry sky" appearance** (Figure 28-8 ■). The "sky" represents the blue nuclei of the neoplastic lymphocytes; the "stars" are formed by scattered pale-staining tingible body macrophages. The infiltrating lymphoid cells are intermediate in size with nuclei approximately the same size as those of the tingible body macrophages. Multiple small nucleoli are usually present, and mitotic figures and apoptotic bodies are frequent. The latter two features are characteristic of high-grade lymphoma.

The differential diagnosis for Burkitt lymphoma includes B lymphoblastic leukemia/lymphoma and DLBCL. The presence of surface immunoglobulin and absence of terminal deoxynucleotidyl transferase (TdT) allow distinction of Burkitt lymphoma from B lymphoblastic leukemia/lymphoma (Chapter 27). Distinguishing Burkitt lymphoma from DLBCL is often more difficult because the two can share the same immunophenotype: CD19+, sIg+, CD10+, CD5−.

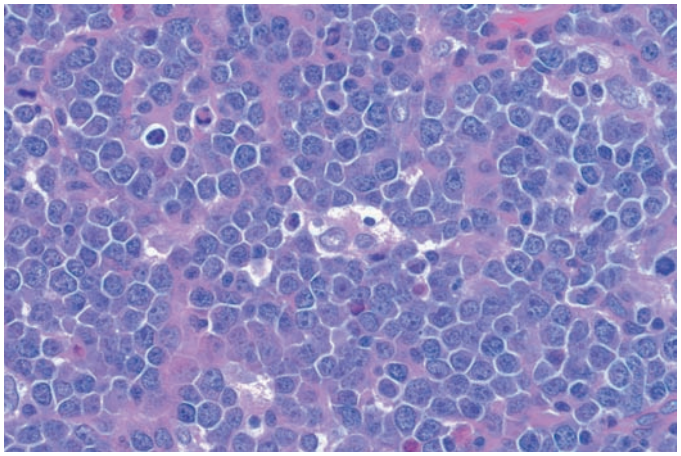


FIGURE 28-8 Burkitt lymphoma. Intermediate size lymphocytes with multiple nucleoli and scant cytoplasm. Numerous mitotic figures are present. The presence of apoptotic bodies indicates individual cell necrosis. There is a "starry-sky" appearance because of pale-staining tingible body macrophages scattered in an infiltrate that appears basophilic because of staining of the tumor cell nuclei (lymph node biopsy, H&E stain, 100× magnification).

Most cases of Burkitt lymphoma have an isolated chromosome translocation leading to rearrangement of the *MYC* and *IGH* genes, typically t(8;14) or less frequently t(2;8) or t(8;22). *MYC* gene rearrangement occurs in only 5% of DLBCL. This distinction is important because the therapeutic regimen used for Burkitt lymphoma usually differs from that used for DLBCL. With the appropriate aggressive multiagent chemotherapy, Burkitt lymphoma is potentially curable.

Plasma Cell Neoplasms

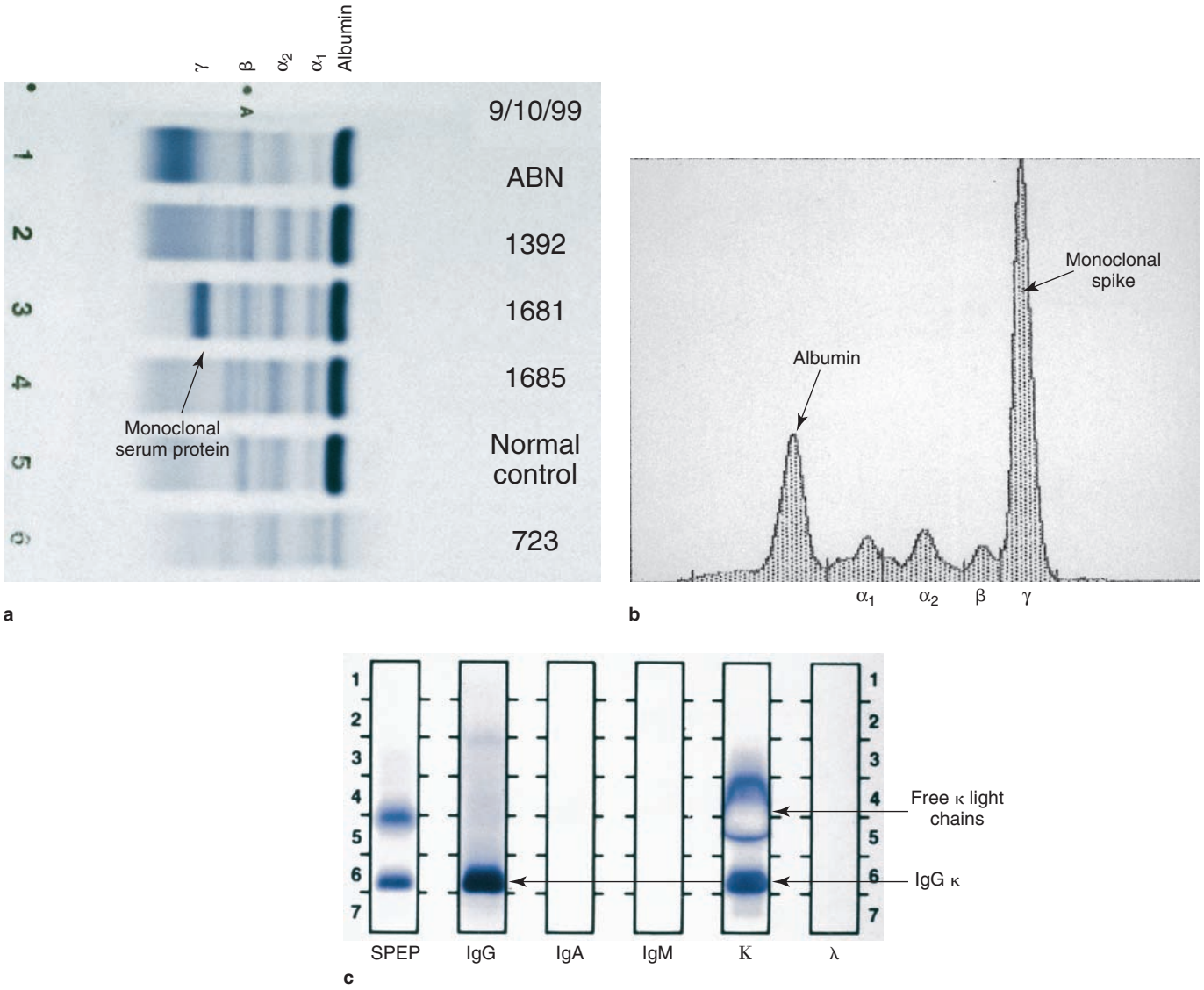
The **plasma cell neoplasms** are considered to be a group of diseases composed of immunoglobulin-secreting cells in the absence of neoplastic B lymphocytes. It is important to distinguish this group of neoplasms from the mature lymphoid neoplasms that can demonstrate plasmacytic differentiation (e.g., LPL). Plasma cell neoplasms are diverse disorders that rarely involve lymph nodes and usually secrete monoclonal immunoglobulin into the serum and/or urine. Serum or urine protein electrophoresis (SPEP or UPEP) reveal increased protein with a narrow range of electrophoretic mobility (M spike) (Figure 28-9a, b ■). The M spike can be further characterized by immunofixation electrophoresis (IFE) to confirm the presence of a monoclonal protein and determine the immunoglobulin class (Figure 28-9c). The monoclonal protein usually contains one immunoglobulin light chain (κ or λ) and one heavy chain with the following incidence: IgG > IgA > IgM > IgD > IgE. Normal immunoglobulin production is usually decreased, leading to functional hypogammaglobulinemia. Plasma cell neoplasms can produce an excess of immunoglobulin light chains, light chains only, heavy chains only, or no immunoglobulin (nonsecretory). Patients with light chain only disease can have a normal SPEP because the protein passes into the urine. The presence of free immunoglobulin light chains in the urine is referred to as **Bence-Jones proteinuria**.

The plasma cell neoplasms can be divided into disease entities based on the distribution and extent of disease (solitary versus multiple lesions and bone versus extraosseous) and the characteristics of the immunosecretory protein produced (class of heavy chain and the presence of amyloid production, immunoglobulin heavy, or light chain) (Table 28-4 ★ and Table 28-5 ★).

Plasma Cell Myeloma

Plasma cell myeloma is a plasma cell neoplasm that usually has diffuse bone marrow involvement. Several clinical variants range from asymptomatic to an aggressive disease that is identified on the presentation, radiologic findings, and laboratory information. Symptomatic plasma cell myeloma is characterized by an M protein in the urine or serum, clonal plasma cells in the bone marrow whose symptoms are hyper**c**alcemia, **r**enal insufficiency, **a**nemia, lytic **b**one lesions (CRAB), and evidence of related organ or tissue impairment. Patients often present with bone pain and/or pathologic fractures because of tumor infiltration. Asymptomatic (smoldering) myeloma is characterized by M protein in serum at a level >30 g/L and/or 10% or more of clonal plasma cells in the bone marrow but no organ or tissue impairment or symptoms. This smoldering form is distinguished from monoclonal gammopathy of undetermined significance by the requirement for 10% or more clonal plasma cells in the bone marrow.

Cytogenetic abnormalities can be found in >90% of patients if FISH is used. There are two genetic subtypes based on chromosome content: hyperdiploid and nonhyperdiploid.[9] The hyperdiploid group includes trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. The nonhyperdiploid group is characterized by reciprocal translocations, deletions, and complex karyotypes. The most frequent translocation involves the immunoglobulin heavy chain variable region on chromosome 14q32 and involves cyclin D1. Examination of either the bone marrow from a lytic lesion or the posterior iliac crest reveals an abnormal infiltrate of plasma cells replacing the normal hematopoietic cells (Figure 28-10a, b ■). For symptomatic plasma cell myeloma, there is no requirement on the proportion of bone marrow plasma cells. Although neoplastic plasma cells can appear normal, abnormal forms with more finely distributed chromatin or nucleoli are often present. In addition, abnormal plasma cells can contain intranuclear inclusions composed of immunoglobulin (**Dutcher bodies**) (Figure 28-11a ■) or red-tinged cytoplasm (**flame cells**) (Figure 28-11b). Although intracytoplasmic inclusions composed of immunoglobulin are also seen, either as single inclusions (Russell body) or multiple inclusions (Mott cell) (Figure 28-11c), they can also be seen in reactive plasma cells. Neoplastic plasma cells rarely circulate in the blood, but the peripheral smear is often abnormal because of stacking of the erythrocytes (**rouleaux** formation) (Figure 28-11d). In addition,



■ **FIGURE 28-9** Monoclonal gammopathy. **(a)** Serum protein electrophoresis. Sample 1681 displays a band in the early gamma region. **(b)** Densitometry scan reveals a "spike" in the gamma region. A similar spike was found in the urine. **(c)** Immunofixation electrophoresis performed on a urine sample reveals two monoclonal bands composed of IgG kappa and free kappa light chains (Bence-Jones proteins).

★ **TABLE 28-4** Plasma Cell Neoplasms

Plasma cell myeloma	Symptomatic
	Asymptomatic (smoldering)
	Osteosclerotic
Monoclonal gammopathy of undetermined significance (MGUS)	
Plasmacytoma	Solitary plasmacytoma of bone
	Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition disease	Primary amyloidosis
	Light and heavy chain deposition disease

★ **TABLE 28-5** Key Features of Selected Plasma Cell Neoplasms

Neoplasm	Features
Symptomatic myeloma	M spike in serum or urine
	Clonal plasma cells in bone marrow
	Related organ or tissue damage (CRAB)
Asymptomatic myeloma	M spike in serum or urine (>30 g/L)
	Clonal plasma cells in bone marrow >10%
	No related organ or tissue damage
Monoclonal gammopathy of undetermined significance (MGUS)	M spike in serum or urine (<30 g/L)
	Clonal plasma cells in bone marrow <10%
	No related organ or tissue damage
Plasmacytoma	Solitary collection of clonal plasma cells
	Can have M spike
	No clonal plasma cells elsewhere in bone marrow
	No related organ or tissue damage

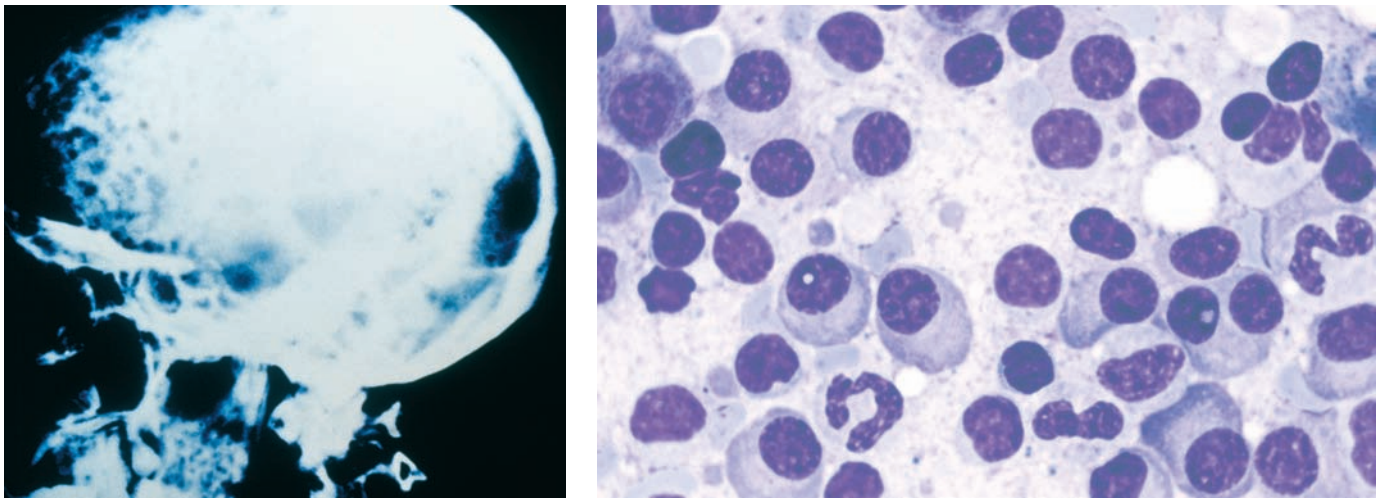
CRAB = hypercalcemia, renal insufficiency, anemia, bone lesions

the stained blood smear often has a blue background because of increased serum protein.

Patients under 65 years of age can be treated with high-dose chemotherapy followed by autologous or allogeneic stem cell transplant. Those over the age of 65 can be treated with chemotherapy (melphalan) and prednisone. The prognosis of symptomatic plasma cell myeloma is poor with a median survival of 3–4 years. Patients with asymptomatic plasma cell myeloma can have stable disease for many years but often progress to symptomatic myeloma.

Monoclonal Gammopathy of Undetermined Significance

Identification of a monoclonal spike is not sufficient to diagnose plasma cell myeloma. The term **monoclonal gammopathy of undetermined significance (MGUS)** is used to describe a low level of serum monoclonal protein without evidence of an overt neoplasm. Therefore, MGUS is a diagnosis that requires exclusion of other plasma cell and lymphoid malignancies. The prevalence of a monoclonal serum spike increases with age and is present in 3% of asymptomatic patients over 70 years of age. There is no treatment for MGUS.



a **b**

■ **FIGURE 28-10** Symptomatic plasma cell myeloma. **(a)** Skull x-ray demonstrating multiple lytic bone lesions giving a "moth-eaten" appearance. **(b)** Replacement of bone marrow hematopoietic precursors by an infiltrate of plasma cells (bone marrow aspirate, Wright stain, 1000× magnification).

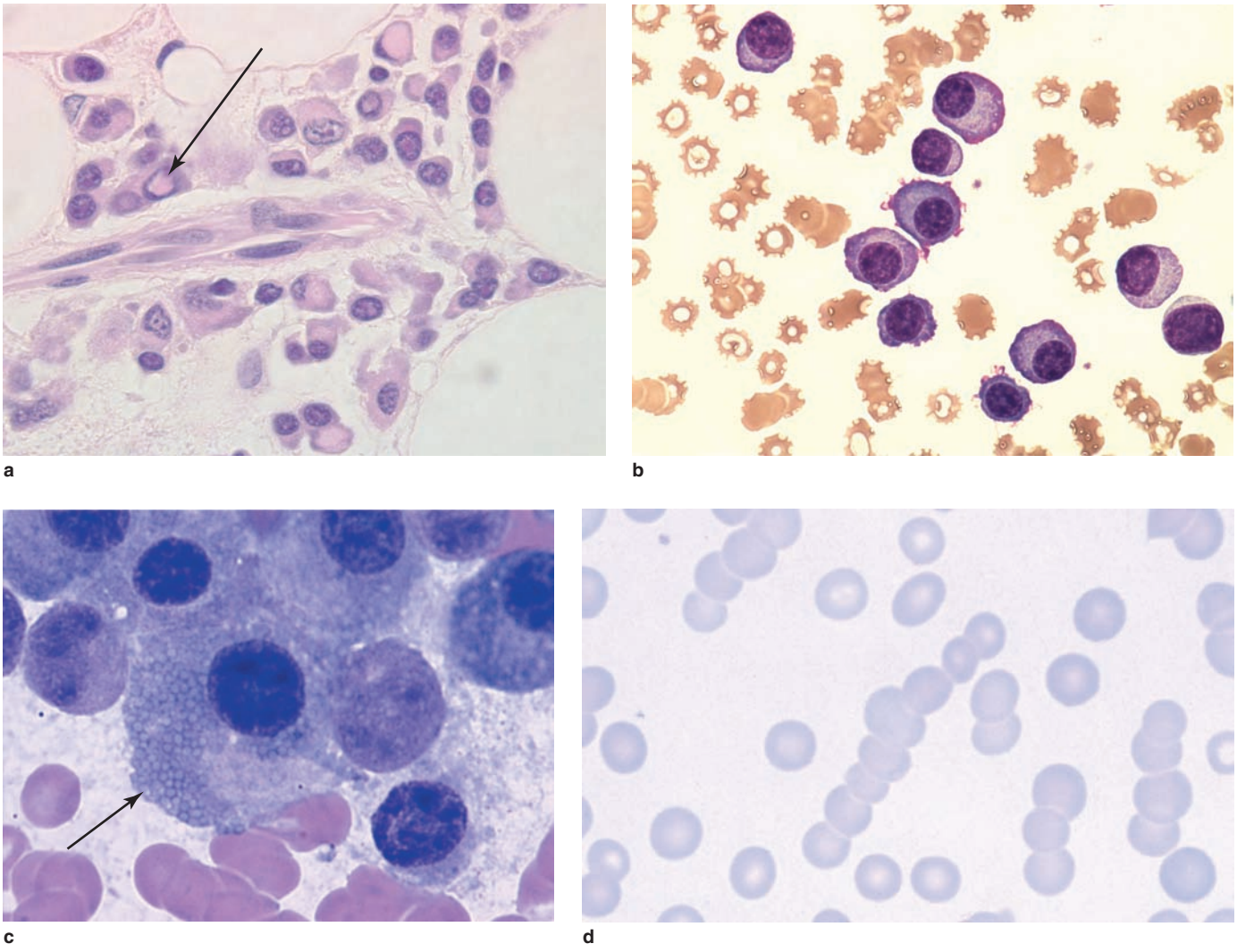


FIGURE 28-11 Symptomatic plasma cell myeloma. **(a)** Plasma cell with intranuclear inclusion indicated with arrow (Dutcher body) (H&E stain, 1000× magnification). **(b)** Many plasma cells with a red tinge to the cytoplasm imparting a flame-cell appearance (bone marrow, Wright stain, 1000× magnification). **(c)** Plasma cell with multiple cytoplasmic inclusions (Mott cell) (bone marrow, Wright stain, 1000× magnification). **(d)** Stacking of peripheral blood erythrocytes resulting from the presence of increased immunoglobulin (Rouleaux) (peripheral blood, Wright stain; 1000× magnification).

Although most patients have stable disease and die of other causes, MGUS can progress to an overt plasma cell neoplasm.

Plasmacytoma

A **plasmacytoma** is a localized, tumorous collection of clonal plasma cells. The prognosis of a plasmacytoma is related to its location. Many patients with plasmacytoma of bone go on to develop additional plasmacytomas and ultimately meet the criteria for symptomatic plasma cell myeloma. In contrast, plasmacytoma of extraosseous locations, such as the upper respiratory tract, have a good prognosis with only rare recurrence after excision and a low incidence of dissemination. Sometimes distinguishing extraosseous plasmacytoma from MALT lymphoma with plasmacytic differentiation is difficult.

MATURE T- AND NK-CELL NEOPLASMS

T-Cell Prolymphocytic Leukemia

T-cell prolymphocytic leukemia (T-PLL) is a rare disorder of adults that, like B-PLL, usually presents with marked lymphocytosis and splenomegaly. However, patients with T-PLL have more frequent lymphadenopathy, hepatomegaly, and skin lesions (infiltration)

than do patients with B-PLL. In addition, the morphologic appearance of T-PLL is more variable than that of B-cell PLL. The cells of T-PLL often have a prominent nucleolus but can be medium in size or small and can have convoluted nuclear outlines. T-PLL has a mature phenotype with expression of CD3, CD2, CD5, and CD7. Most cases are CD4+. Like T lymphoblastic leukemia, some cases of T-PLL express both CD4 and CD8, but PLL is negative for TdT. The majority of cases of T-PLL have cytogenetic abnormalities, most frequently inv(14)q11q32, del(11q), i(8q), and trisomy 8q. T-PLL is an aggressive disorder with a median survival time of only 7.5 months, although some response has been seen with anti-CD52 monoclonal antibody therapy.

T-Cell Large Granular Lymphocytic (T-LGL) Leukemia

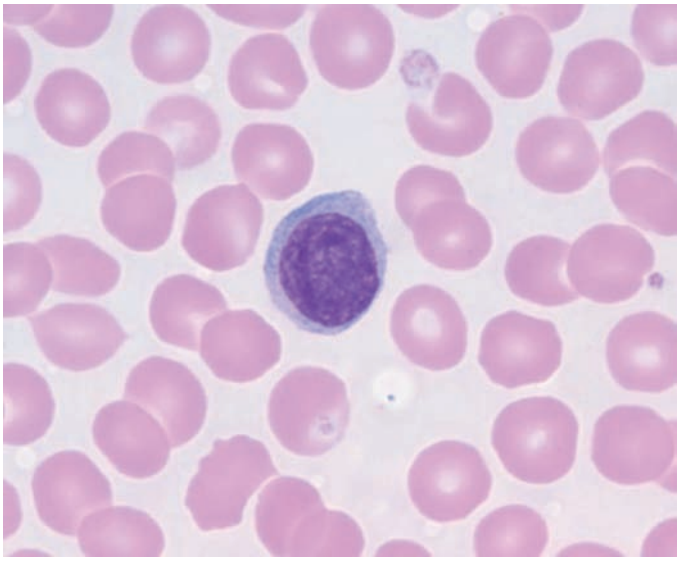
T-cell large granular lymphocytic (T-LGL) leukemia often has a presentation similar to chronic lymphocytic leukemia, but is composed of mature T cells. T-LGL is characterized by a modest lymphocytosis composed of cells with abundant pale-staining cytoplasm, azurophilic cytoplasmic granules, and nuclei with mature clumped chromatin. Large granular lymphocytes are normal components of the peripheral blood and are usually NK-like T cells (CD3+) or NK cells (CD3−) (Chapter 8). Distinction of T-LGL leukemia from reactive lymphocytosis requires using molecular diagnostic or cytogenetic studies to demonstrate an abnormal phenotype or evidence of clonality. T-LGL leukemia must also be distinguished from neoplasms of NK cells, including indolent chronic lymphoproliferative disorders of NK cells and aggressive NK-cell leukemia. The neoplastic cells of T-LGL leukemia are T lymphocytes with the following phenotype: CD2+, CD3+, CD4−, CD5+, CD7+, CD8+, CD16+, CD56−/+, CD57+/− (usually CD56−/CD57+). The cells can demonstrate an abnormal phenotype with loss of pan T-cell antigens. Molecular diagnostic studies can be used to confirm the diagnosis by demonstration of clonal T-cell receptor rearrangement. In addition to lymphocytosis, patients with T-LGL leukemia often have anemia, neutropenia, and thrombocytopenia. Anemia can be related to bone marrow infiltration or aplasia of erythroid precursors (pure red cell aplasia). Neutropenia and thrombocytopenia can be the result of immune destruction, splenic sequestration, or marrow infiltration. Although splenomegaly is common, lymphadenopathy and hepatomegaly are uncommon. Approximately 25% of patients with T-LGL leukemia have symptomatic rheumatoid arthritis, and many more are positive for rheumatoid factor. Therefore, many patients with T-LGL leukemia have the triad (rheumatoid arthritis, splenomegaly, and neutropenia) defining Felty's syndrome. Patients with T-LGL leukemia usually have an indolent course with >80% overall survival after 10 years.

Sézary's Syndrome

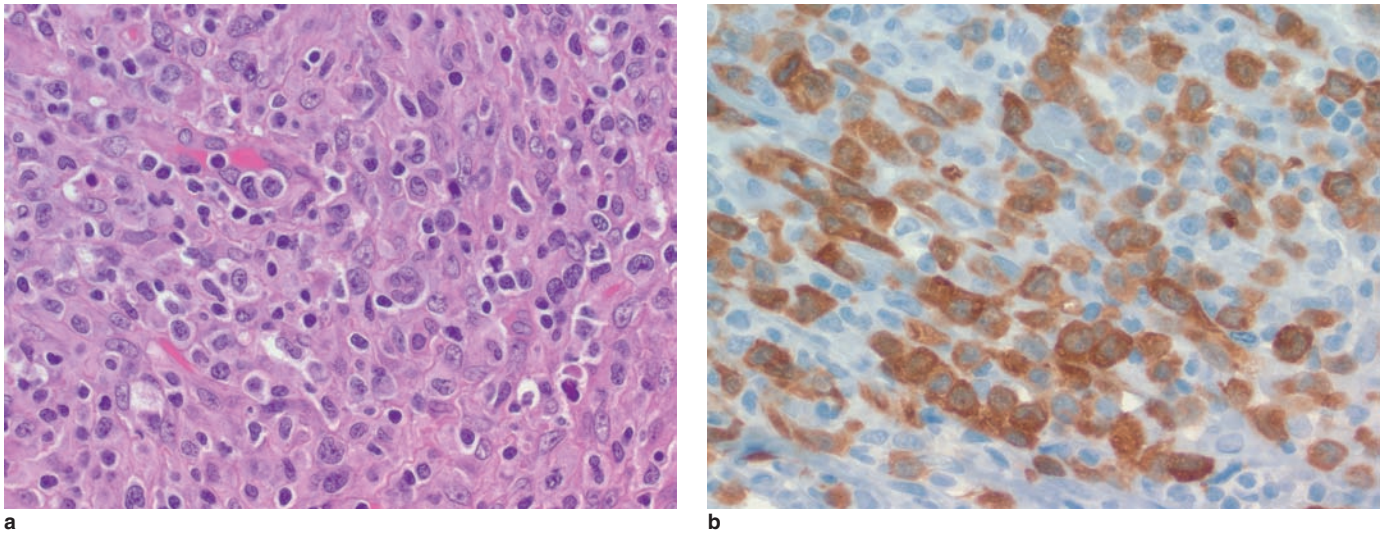
Sézary syndrome is a neoplasm of mature T cells that is defined by the combination of erythroderma (red skin), generalized lymphadenopathy, and clonally related abnormal T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood. **Sézary cells** are neoplastic T lymphocytes that have a very irregular, convoluted (cerebriform) nuclear outline (Figure 28-12 ■). Although the marked nuclear convolutions can be easily identified on electron microscopy, this technique is rarely used for diagnosis. Abnormal cells can be counted in a blood smear leukocyte differential to determine a Sézary count; an absolute count of >1000 cells/mm^3 is required for diagnosis. Sézary cells are mature helper T cells (CD3+, CD4+) but usually lack expression of CD7. Flow cytometric studies can be performed to identify these cells and the presence of a CD4:CD8 ratio of >10, or aberrant antigen expression can be used to support the presence of a neoplasm. Molecular diagnostic studies can be used to confirm the presence of neoplastic cells by demonstrating clonal T-cell receptor gene rearrangement. Sézary syndrome is closely related to the primary cutaneous T-cell lymphoma mycosis fungoides (MF) and has neoplastic cells with a similar phenotype and appearance but differs in clinical presentation and distribution of disease. MF is usually characterized by cutaneous patches and plaques, rather than diffuse erythroderma, and has less frequent blood involvement. Treatment is usually palliative to relieve symptoms and improve the quality of life. Clinical trials with ultraviolet B radiation and high-dose chemotherapy and radiation with stem cell transplant are ongoing.

Anaplastic Large Cell Lymphoma (ALCL)

One of the best-characterized subtypes of T/NK-cell lymphoma is **anaplastic large cell lymphoma**. Most ALCL cases have large bizarre anaplastic cells that can resemble Reed-Sternberg cells and Hodgkin variant cells (Figure 28-13a ■). Like HL and some large cell NHLs, ALCL is usually positive for the CD30 antigen (initially referred to as the *Ki-1 antigen*). However, in contrast to classic HL, ALCL usually lacks CD15 expression and evidence of Epstein-Barr virus infection and is often positive for leukocyte common antigen



■ **FIGURE 28-12** Sézary syndrome. Abnormal large lymphocyte with relatively finely distributed chromatin and numerous nuclear folds (Sézary cell) (peripheral blood, H&E stain, 1000× magnification).



a **b**

■ **FIGURE 28-13** Anaplastic large cell lymphoma. **(a)** Abnormal infiltrate of large cells with eosinophilic cytoplasm, including cells with multilobated nuclei (lymph node biopsy, H&E stain, 100× magnification). **(b)** ALK expression (lymph node biopsy, immunohistochemical stain, 200× magnification).

(LCA) and epithelial membrane antigen (EMA). Although ALCL is derived from T cells, frequent loss of T-cell antigen expression can result in a null phenotype (lack of expression of either T- or B-cell antigens). Most, but not all, ALCL cases involving lymph nodes contain the translocation t(2;5) that joins the nucleophosmin (*NPM1*) and anaplastic large-cell kinase (*ALK*) genes. This leads to abnormal expression of ALK protein that can be detected by immunohistochemistry (Figure 28-13b). The presence of ALK protein expression can assist in the distinction of ALCL from Hodgkin lymphoma and most other subtypes of NHL. However, absence of staining for ALK protein does not exclude a diagnosis of ALCL. Most cases of cutaneous ALCL are ALK negative. Primary cutaneous ALCL can be treated successfully by local excision or radiation therapy. Approximately 70% of patients with systemic ALCL go into remission with systemic multiagent chemotherapy, but the rate of relapse is high.

Peripheral T-Cell Lymphoma, Not Otherwise Specified (NOS)

NHL with a mature T-cell or NK-cell (T/NK) phenotype represents only 15% of lymphomas in the United States. Several subtypes of peripheral T/NK-cell lymphoma are defined using a combination of parameters including the clinical presentation, distribution of disease (nodal or extranodal), association with Epstein-Barr virus, and immunophenotype (CD4+ or CD8+, and presence of NK-associated or cytotoxic markers). However, a large category of peripheral T-cell lymphoma (30% in Western countries) cannot be put into one of the other subtypes in the current classification. This category is classified as peripheral T-cell lymphoma, not otherwise specified.

Although it has the morphologic appearance of peripheral T/NK-cell lymphoma, NOS is quite variable, and a number of morphologic features suggest this diagnosis. The tumor cells vary in size and can display abundant clear or pale-staining cytoplasm and irregular nuclear outlines. The infiltrate is often heterogeneous with histiocytes, plasma cells, and eosinophils in addition to the neoplastic cells. However, immunophenotyping is essential for distinction from B-cell NHL and, in some cases, HL. Peripheral T/NK-cell lymphoma, NOS demonstrates expression of pan-T-cell antigens with frequent abnormal lack of staining for one or more of the expected T-cell antigens (e.g., CD5− and/or CD7−). The presence of an abnormal immunophenotype or clonal rearrangement of the T-cell receptor can assist in the distinction of T-cell lymphoma from a reactive process. Nodal cases usually show a CD4+/CD8− phenotype. CD4/CD8 double positivity or double negativity can be seen. Cytogenetics usually reveals a complex karyotype. The normal cell counterpart is thought to be the activated, mature, CD4+ T lymphocyte.

Peripheral lymph node enlargement with node involvement is usually present. Other sites can be involved including bone marrow, spleen, and liver. In general, peripheral T-cell lymphoma is an aggressive disease with a poor response to therapy and frequent relapses. However, some patients are cured by combination chemotherapy.

HODGKIN LYMPHOMA (HL)

HL differs from NHL in its clinical presentation and histologic appearance. Separation of these two broad categories of lymphoma is important because they are treated with different combinations of chemotherapeutic agents. HL is composed of large tumor cells that do not resemble a normal cell counterpart and are accompanied by many reactive cells. HL has several histologic subtypes that differ in phenotype, appearance of large neoplastic cells, and nature of reactive component. The main histologic types are lymphocyte-predominant (LP) HL and classic HL. Classic HL is further classified into nodular sclerosis (NS), mixed cellularity (MC), lymphocyte-rich (LR), and lymphocyte depletion (LD) subtypes (Table 28-6 ★).

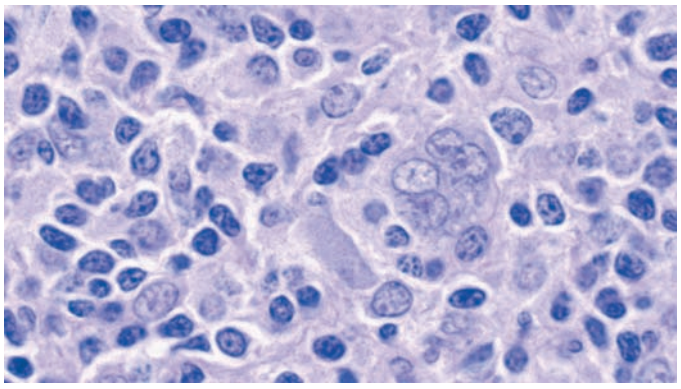
★ **TABLE 28-6** Classification of Hodgkin Lymphoma

Subtype	Sclerosis	Lymphocytes	Tumor Cells	Variants	Cell Type
LP	−	++++	+	L&H	B-cell
NS	Present	++	++	Lacunar	?
MC	−	++	++	−	?
LR	−	++++	+	−	?
LD	−/+	+	++++	−	?

NS = nodular sclerosis classical HL; MC = mixed cellularity classical HL; LD = lymphocyte depletion classical HL; LR = lymphocyte-rich classic HL; LP = lymphocyte-predominant HL; L&H = malignant cells characteristic of LP HL; ? = uncertain cell of origin, + = few; ++++ = many

LP HL is composed of numerous reactive small lymphocytes and rare tumor cells that often grow in loose nodular aggregates. The tumor cells in LP HL (L&H cells) characteristically have a large multilobated nucleus with delicate nuclear membranes, finely granular chromatin, and small indistinct nucleoli (**popcorn cells [L&H cells]**) (Figure 28-14 ■). Unlike the neoplastic cells of classical HL, those of LP HL have a B-cell phenotype: LCA+, CD45+, CD20+, CD30−/+, CD15− (Table 28-6). Therefore, the phenotype of LP HL overlaps that of B-cell NHL. The reactive small lymphocytes that surround the neoplastic cells of LP HL often include an unusual subset of CD57 positive T cells.

The tumor cells in classical HL have large prominent eosinophilic nucleoli and coarse nuclear membranes. One variant of tumor cell is found in all cases of classic HL: the **Reed-Sternberg (R-S) cell**, which has two or more nuclear lobes containing inclusion-like nucleoli and an area of perinucleolar clearing imparting an owl's eye appearance (Figure 28-15a ■). The reactive cells accompanying the neoplastic cells of classical HL include a heterogeneous mixture of small lymphocytes, histiocytes, eosinophils, and plasma cells. The tumor cells of classical HL are negative for LCA and ALK but positive for the CD30 antigen. They can also be positive for CD15 and/or EBV.



■ **FIGURE 28-14** Nodular lymphocyte predominant Hodgkin lymphoma. Large L&H ("popcorn") cell with multilobated nucleus, delicate nuclear membranes, and small basophilic nucleoli. The background contains many histiocytes with pale-staining eosinophilic cytoplasm and small lymphocytes (H&E stain, 200× magnification).

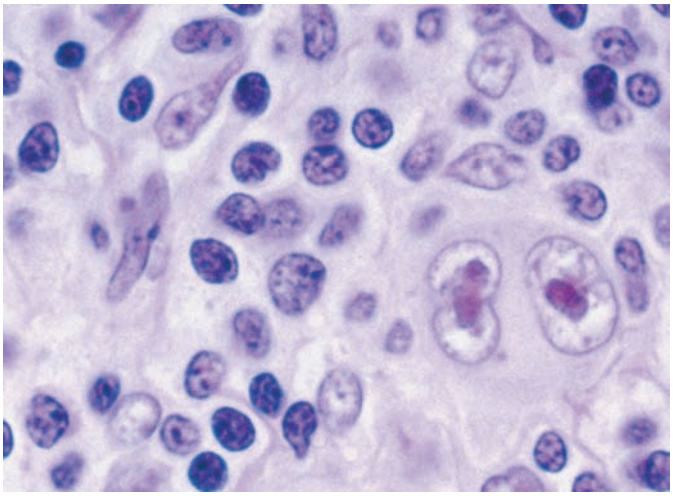
☑ CHECKPOINT 28-4

Name and describe the cell that is characteristic of Hodgkin lymphoma.

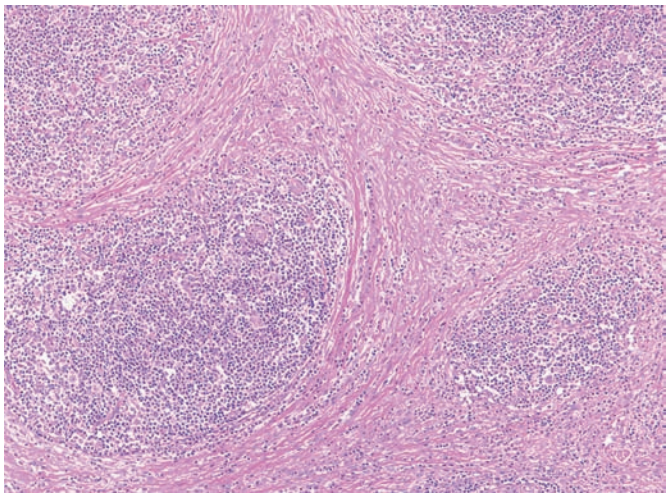
Classical HL is further divided into histologic subtypes based on the presence of sclerosis (fibrosis), type of tumor cell variants, and proportion of reactive lymphocytes. The NS subtype of HL is characterized by C-shaped bands of birefringent fibrous tissue (Figure 28-15b), nodular aggregates of cells, and tumor cell variants with cytoplasmic clearing and delicate, multilobated nuclei (**lacunar cells**) (Figure 28-15c). The LR form of classical HL has a background of reactive cells including many small lymphocytes, but in contrast to LP HL, the neoplastic cells have the phenotype of classical HL. LD HL is a rare subtype that has many tumor cells and only rare reactive lymphocytes. A diagnosis of MC HL is made after excluding the other classic subtypes: NS, LR, and LD. However, the histologic subtypes of classical HL do not have independent prognostic significance. The stage of HL is one of the most important prognostic markers. The 5-year survival for patients with Stage I or IIA Hodgkin lymphoma currently is approximately 90% and for Stage IV is 60–70%. Treatment options depend on the stage of disease and can include chemotherapy with or without radiation.[10]

☑ CHECKPOINT 28-5

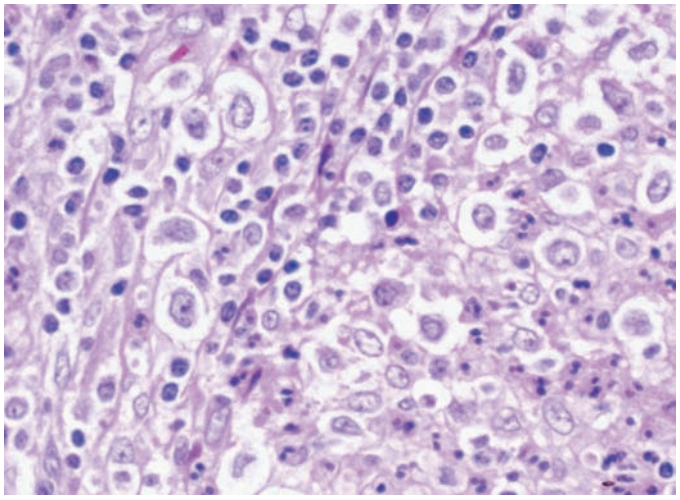
What clinical finding differentiates multiple myeloma from other plasma cell neoplasms?



a



b



c

■ **FIGURE 28-15** Hodgkin lymphoma, classic type, nodular sclerosis subtype. **(a)** Reed-Sternberg cell with two nuclear lobes and prominent, eosinophilic nucleoli giving an owl's eye appearance. The background contains many small lymphocytes and a few pale eosinophilic histiocytes (lymph node biopsy, H&E stain, 200× magnification). **(b)** Bands of fibrous tissue isolate nodules containing an abnormal cellular infiltrate (nodular sclerosing pattern) (lymph node biopsy, H&E stain, 20× magnification). **(c)** Lacunar cells with abundant clear-staining cytoplasm, delicate nuclear membranes, and small basophilic nucleoli (lymph node biopsy, H&E stain, 100× magnification).

Summary

The mature lymphoid neoplasms represent a diverse group of neoplasms that vary in clinical presentation, morphologic appearance, immunophenotype, and genotype. The WHO classifies these neoplasms into mature B-cell and mature T/NK-cell neoplasms. As in the precursor B-cell and T-cell neoplasms, the leukemic entities are characterized by peripheral blood and bone marrow involvement whereas the lymphomas have tumorous masses involving the lymph nodes and other lymphoid tissue. The malignant cells often resemble one or more stages of normal lymphocyte development and, therefore, must be distinguished from reactive proliferations of these cells. The diagnosis usually requires a combination of conventional morphology, immunophenotyping, molecular analysis, and cytogenetics. HL differs from other lymphomas clinically and histologically; it is composed of large tumor cells that do not resemble a normal cell counterpart and are accompanied by many reactive cells. HL has several histologic subtypes that differ in phenotype, appearance of the large neoplastic cells, and nature of the reactive component. Although the classification of lymphoid malignancies is complicated, it is important to separate distinct disease entities that have a predictable outcome and response to specific therapy.

Review Questions

Level I

1. Which of the following is the most likely distribution of disease in a patient with leukemia? (Objective 3)
   A. lytic bone lesion
   B. tumorous mass involving lymph nodes
   C. tumorous mass involving the tonsil
   D. widespread involvement of the bone marrow
2. A 60-year-old male being evaluated for insurance purposes was found to have lymphocytosis. The peripheral smear revealed a uniform population of small mature lymphocytes. Which of the following is helpful in differentiating a benign from a malignant lymphocytosis? (Objectives 8, 9)
   A. cytochemistry
   B. immunophenotyping
   C. morphology
   D. absolute lymphocyte count
3. Which of the following indicate(s) the presence of a mature lymphoid neoplasm? (Objective 2)
   A. clonal immunoglobulin gene rearrangement
   B. population of lymphocytes expressing only one immunoglobulin light chain
   C. population of lymphocytes expressing an abnormal phenotype
   D. all of the above
4. A patient with chronic lymphocytic leukemia had a complete blood count (CBC) with differential. The differential showed 20% nucleated cells similar to the ones in Figure 28-2. This indicates: (Objective 8)
   A. transformation of CLL to ALL
   B. development of hairy cell leukemia
   C. Richter's transformation
   D. prolymphocytic transformation of CLL
5. You are asked to perform a peripheral blood smear review on a 56-year-old male with a history of lytic bone lesions, hypercalcemia, and a recent diagnosis of plasmacytoma. Which of the following is the most likely peripheral blood finding? (Objective 10)
   A. plasmacytosis
   B. lymphocytosis
   C. agglutination
   D. rouleaux
6. A bone marrow biopsy was performed on a patient with bone pain. The bone marrow differential revealed 40% plasma cells. Serum electrophoresis showed an increased protein with an M spike. What would you expect to find on the peripheral blood smear in this patient? (Objectives 3, 10)
   A. rouleaux
   B. spherocytes
   C. plasma cells
   D. >20% blasts
7. The WHO classification of lymphoid neoplasms recommends which of the following studies to identify neoplastic cells and type of lymphoid malignancy? (Objective 9)
   A. genotype
   B. morphology
   C. phenotype
   D. all of the above
8. The Reed-Sternberg cell is found in what type of lymphoid malignancy? (Objective 6)
   A. chronic lymphocytic leukemia
   B. multiple myeloma
   C. HL
   D. NHL
9. A peripheral smear performed on a 35-year-old male with lymphocytosis reveals numerous smudge cells. Which of the following does this finding indicate? (Objectives 3, 8)
   A. chronic lymphocytic leukemia
   B. infectious mononucleosis
   C. lymphoblastic leukemia
   D. none because the finding is not diagnostic
10. Peripheral smear examination of a patient with chronic lymphocytic leukemia reveals 8% prolymphocytes. This finding is consistent with which of the following diagnoses? (Objective 8)
    A. chronic lymphocytic leukemia
    B. prolymphocytic leukemia
    C. prolymphocytoid transformation of CLL
    D. Richter's transformation

Level II

1. Which of the following is(are) involved in the pathogenesis of low-grade follicular lymphoma? (Objective 8)
   A. genes involved in apoptosis
   B. inherited immunodeficiency
   C. proto-oncogenes
   D. tumor suppressor genes
2. Which type of lymphoma can antimicrobial therapy cure? (Objective 8)
   A. Burkitt lymphoma
   B. Hodgkin lymphoma
   C. MALT lymphoma
   D. HIV-associated non-Hodgkin lymphoma
3. A lymph node biopsy is performed on a patient with lymphadenopathy. Which of the following findings is(are) characteristic of a reactive proliferation rather than malignant lymphoma? (Objective 9)
   A. a mixed population of cells varying in size, shape, and color
   B. clonality of cells
   C. presence of large, bizarre cells
   D. many cells with mitotic activity
4. A CBC performed on a 65-year-old female reveals lymphocytosis. A bone marrow reveals replacement of the hematopoietic precursors by an infiltrate of mature lymphocytes. Which of the following is the most likely course of the disease? (Objective 5)
   A. cure following therapy
   B. rapid progression
   C. slow progression
   D. spontaneous remission
5. Which of the following phenotypes is characteristic of chronic lymphocytic leukemia? (Objective 1)
   A. CD19+, CD5+, CD23+
   B. CD19+, CD5−, CD23+
   C. CD19+, CD5+, CD23−
   D. CD19+, CD5−, CD23−
6. A CBC performed on an 80-year-old female reveals an absolute lymphocyte count of 8×10^9/L. Examination of the peripheral blood smear reveals a uniform population of small lymphocytes. Which of the following procedures is most likely to establish a diagnosis? (Objectives 1, 5)
   A. cytogenetics
   B. flow cytometry immunophenotyping
   C. immunoglobulin gene rearrangement
   D. lymph node biopsy
7. A patient presents with pancytopenia and massive splenomegaly but no lymphadenopathy. Unusual cells noted on the blood smear had abundant pale-staining cytoplasm and cytoplasmic projections. The nuclei were oval with a fine chromatin pattern. What cytochemical stain would be helpful in identifying these cells? (Objective 5)
   A. acid phosphatase stain after incubation with tartrate
   B. periodic acid-Schiff
   C. Sudan black B
   D. myeloperoxidase
8. A CBC performed on a 60-year-old male with a history of rheumatoid arthritis revealed neutropenia and an absolute lymphocytosis. Examination of the peripheral smear revealed many lymphocytes with abundant pale-staining cytoplasm and cytoplasmic granules. Which of the following is the most likely phenotype? (Objectives 1, 5)
   A. CD3+, CD2+, CD57+
   B. CD19+, CD5+, CD57−
   C. CD2+, CD3−, CD56+
   D. CD19+, CD10+, CD34+
9. A patient previously diagnosed with SLL is found to have peripheral blood lymphocytosis. The lymphocytes are small and have round nuclei with clumped chromatin. Which of the following is the most appropriate interpretation? (Objective 3)
   A. This is a different presentation (CLL) of the same disease process.
   B. The patient has a new lymphoid neoplasm: chronic lymphocytic leukemia.
   C. The lymphoma has transformed.
   D. The patient has developed a therapy-related neoplasm.
10. A lymph node biopsy revealed an infiltrate of small lymphoid cells with a vaguely nodular growth pattern. Flow cytometry revealed a monoclonal population of B cells expressing CD5. Immunohistochemistry revealed nuclear staining for cyclin-D1 protein. Which of the following translocations is associated with these neoplastic cells? (Objective 3)
    A. t(11;14)
    B. t(15;17)
    C. t(1;14)
    D. t(8;14) follicular lymphoma

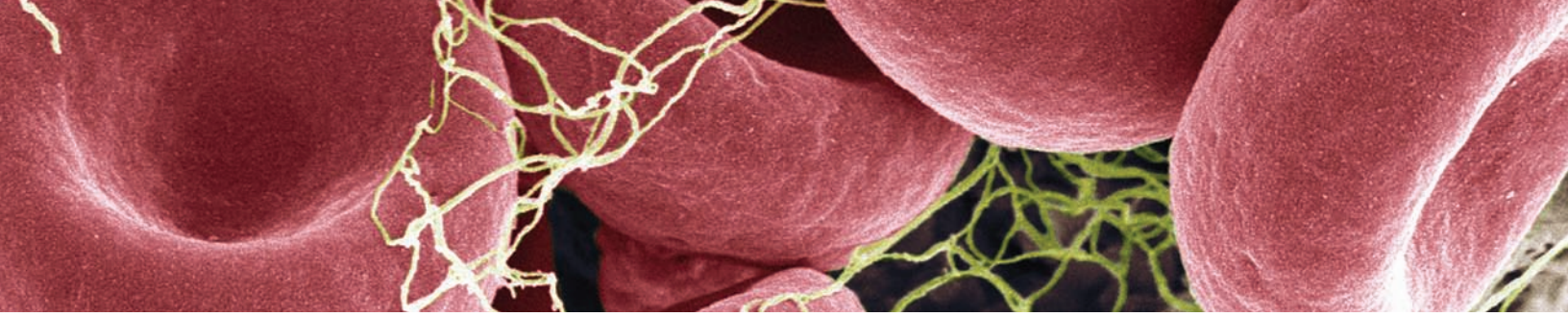
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2008.
2. Alapat D, Coviello-Malle JM, Owens R et al. Diagnostic usefulness and prognostic impact of CD200 expression in lymphoid malignancies and plasma cell myeloma. *Am J Clin Pathol*. 2012;137(1):93–100.
3. Hallek M. Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. *Am J Hematol*. 2013;88:803–16.
4. Verma S, Greaves WO, Ravandi F et al. Rapid detection and quantitation of BRAF mutations in hairy cell leukemia using a sensitive pyrosequencing assay. *Am J Clin Pathol*. 2012;138:153–56.
5. Langabeer SE, O'Brien D, Liptrot S et.al. Correlation of the BRAF V600E mutation in hairy cell leukaemia with morphology, cytochemistry and immunophenotype. *Int J Lab Hematol*. 2012;34:417–21.
6. Blombery PA, Wong SQ, Hewitt CA et al. Detection of BRAF mutations in patients with hairy cell leukemia and related lymphoproliferative disorders. *Haematologica*. 2012;97:780–83.
7. Skarbnik AP, Smith MR. Therapies for mantle cell lymphoma: current challenges and a brighter future. *Discov Med*. 2013;15(82):177–87.
8. Gisbert JP, Calvet X. Review article: common misconceptions in the management of *Helicobacter pylori*-associated gastric MALT-lymphoma. *Aliment Pharmacol Therap*. 2011;34:1047–62.
9. Chesi M, Bergsagel PL. Molecular pathogenesis of multiple myeloma: basic and clinical updates. *Int J Hematol*. 2013;97:313–23.
10. National Cancer Institute. Adult Hodgkin Lymphoma Treatment (PDQ®). www.cancer.gov/cancertopics/pdq; accessed January 5, 2014.



29 Hematopoietic Stem Cell Transplantation

AAMIR EHSAN, MD
ANDREA YUNES, MD
SARA TAYLOR, PHD
SALLY LEWIS, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe the sources of hematopoietic stem cells and characteristics of each source.
2. Identify the diseases that can be treated with different sources of hematopoietic stem cells.
3. List characteristics of stem cells.
4. Describe the significance of ABO and HLA antigens for stem cell transplant.
5. Identify the infections that are serious complications during the peritransplant period.
6. Describe the significance and effects of graft-versus-host disease (GVHD), and compare them to those of the graft-versus-leukemia (GVL) process.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Summarize the collection and processing of hematopoietic stem cells, and assess the success of these procedures.
2. Explain the role of the clinical laboratory professional in stem cell transplantation.
3. Select and outline methods to enumerate hematopoietic stem cells.
4. Explain the complications of stem cell transplant, and assess the patient's risk of developing them.
5. Formulate the sequence of events for a patient who will receive a stem cell transplant.
6. Select laboratory tests used to determine engraftment and assess engraftment given the results obtained by these tests.
7. Differentiate between the types of stem cell transplantation (SCT), and select the most appropriate type for the patient's clinical condition.

Chapter Outline

Key Terms

Allogeneic
Apheresis
Autologous
Chimerism
Clonogenic
Conditioning regimen
Cryopreserved
Engraftment
Graft-versus-host disease (GVHD)
Graft-versus-leukemia (GVL)
Polymorphic
Purging
Syngeneic

Background Basics

This chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review the following material before starting this unit of study:

Level I

- Describe the origin and differentiation of hematopoietic cells. (Chapters 2, 3, 4)
- Outline the classification and explain the etiology and pathophysiology of neoplastic hematologic disorders. (Chapter 23)
- Explain the role of chemotherapy and radiotherapy in treatment of neoplastic hematologic disorders. (Chapters 23–28)

Level II

- Describe the role of cytokines and bone marrow microenvironment in maturation and differentiation of hematopoietic stem cells; explain the role of oncogenes in cancer development. (Chapters 2, 4, 23)
- Explain the use of molecular genetic technology and cytogenetics in diagnosis and prognosis of neoplastic hematopoietic disorders. (Chapters 41, 42)
- Correlate subgroups of neoplastic hematologic disorders with laboratory findings and prognosis. (Chapter 23)

CASE STUDY

We will refer to this case study throughout the chapter.

Brandon, a 35-year-old male (weight 80 kg), was recently diagnosed with AML with differentiation and received induction chemotherapy. Day 21 bone marrow reveals no evidence of residual leukemia. Two weeks later, circulating blasts were seen in the peripheral blood. Brandon is being evaluated for a stem cell transplant.

Consider the laboratory's role in evaluating the transplant, collecting and processing the stem cells, and determining engraftment.

OVERVIEW

The use of stem cells in the therapy of neoplastic hematopoietic disorders has become commonplace. The laboratory's role in this therapy is critical and requires that the clinical laboratory professional knows not only the associated laboratory tests but also why the tests are necessary and how they relate to the overall process of stem cell transplantation (SCT). The stem cell transplant procedure is also referred to as *bone marrow transplant*, *peripheral stem cell transplant*, and *umbilical cord blood transplant* depending on the source of the stem cells. This chapter reviews the sources of hematopoietic stem cells (HSCs); the use of allogeneic, autologous, and umbilical cord stem cells in treating a variety of malignant and nonmalignant disorders; the mobilization, collection, and enumeration of mononuclear cells (MNCs) and CD34+ cells; the procedures used to assess transplant success (**engraftment**); and the role of the clinical laboratory professional during this process.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is a recognized therapeutic modality for leukemias, lymphomas, solid organ tumors, and a variety of metabolic and immunologic disorders (Table 29-1 ★). The concept of SCT came from experiments done five decades ago when it was observed that mice given intravenous marrow infusions

★ **TABLE 29-1** Diseases Treatable with Stem Cell Transplantation

Hematologic		Nonhematologic
Neoplastic	**Non-neoplastic**	
Acute lymphoblastic leukemia	Aplastic anemia	Solid organ malignancies (breast and ovarian cancer, neuroblastoma, Wilms' tumor, germ cell)
Acute myeloid leukemia	Paroxysmal nocturnal hemoglobinuria	
Chronic myelogenous leukemia	Thalassemia	Inborn errors of metabolism (e.g., Hurler's syndrome)
Primary (idiopathic) myelofibrosis	Sickle cell disease	Severe combined immunodeficiency disease
Chronic lymphocytic leukemia	Fanconi's anemia	Wiskott-Aldrich syndrome
Myelodysplastic syndrome	Congenital pure red cell aplasia	Congenital leukocyte dysfunction syndromes
Non-Hodgkin lymphoma		Malignant osteopetrosis
Hodgkin lymphoma		
Multiple myeloma		

could overcome lethal doses of radiation.[1,2] Later, in the 1960s, the first successful bone marrow transplant was performed on a leukemia patient using marrow donated by the leukemic patient's brother.

Stem cells can be obtained from the patient (**autologous**), from a genetically dissimilar but human leukocyte antigen (HLA)-matched donor (**allogeneic**), or from an identical twin (**syngeneic**). After the early successful allogeneic bone marrow SCTs using fresh anticoagulated bone marrow from HLA-matched siblings or family members, clinical practice has expanded to include the use of autologous bone marrow stem cells, autologous and allogeneic peripheral blood stem cells (PBSCs), and umbilical cord stem cells. HLA genes control the body's ability to mount an immune response. These genes code for histocompatible antigens found on the surface of essentially all nucleated cells (Chapters 7, 8).

ORIGIN AND DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS

HSCs are rare cells that can be found in the bone marrow and occasionally in the peripheral blood of healthy individuals. Self-renewal and multilineage differentiation are the two distinct biologic properties that make HSCs remarkable. Pluripotent stem cells differentiate into myeloid and lymphoid progenitor cells (Figure 4-3). The common myeloid progenitor cells (CMPs) produce three types of committed progenitor cells that differentiate into erythroid, granulocyte-macrophage, and megakaryocytic cell pathways. The common lymphoid progenitor cells (CLPs) differentiate into T cells, B cells, natural killer cells, and lymphoid dendritic cells. The committed progenitor cells have been called *colony-forming units (CFUs)* because they give rise to colonies of differentiated progeny in vitro (Chapter 4). From these CFUs arise morphologically recognizable precursors of differentiated cells (pronormoblast, myeloblast, monoblast, megakaryoblast) that further differentiate into mature cells. In summary, pluripotent stem cells have the capacity to self-renew and differentiate along multiple lineages. With additional divisions, their progeny differentiate into a single cell lineage and become progressively restricted to proliferation. The progenitor cells and committed cells lack the property of self-renewal.

SOURCES OF HEMATOPOIETIC STEM CELLS AND TYPES OF STEM CELL TRANSPLANTS

HSCs for clinical use can be harvested from the bone marrow, peripheral blood, and umbilical cord blood (UCB).[3] Fetal bone marrow and liver are also rich in stem cells, but ethical issues limit their use. Unlike other tissues and organs destined for transplant, HSCs are not routinely taken from cadavers because in most situations, the stem cell donor receives hematopoietic cytokines for a few days before the stem cells are harvested to maximize stem cell yield.

Allogeneic Stem Cell Transplantation

In the past, the usual source of stem cells has been the bone marrow, but now allogeneic transplants using PBSCs are more common. Allogeneic transplants are often indicated when the disease process involves the patient's own stem cells (Table 29-2 ★). Allogeneic transplant can be performed for therapy of acute leukemias, chronic myelogenous leukemia (CML), aplastic anemia, hemoglobinopathies, immune deficiencies, and metabolic genetic disorders.[4,5]

Three important factors must be considered in order to achieve successful allogeneic SCT. First, adequate numbers of HSCs should be present in the graft so that when infused into the recipient's circulation, engraftment in the marrow microenvironment will occur. Second, the recipient's immune system should tolerate donor stem cells so that graft rejection does not occur. Third, the donor's immune cells should tolerate host tissue to avoid severe **graft-versus-host disease (GVHD)**, which occurs when immunocompetent donor T lymphocytes recognize HLA antigens on the host cells as foreign and initiate cell injury.

Highly incompatible tissues are almost certain to be rejected. Compatibility is based on the immune system's recognition of certain cell markers as "self." The most critical of these cell markers appear to be the HLA molecules, also known as the *major histocompatibility complex (MHC)*. The HLA system is highly **polymorphic** (genetically variable).

Genes present on the short arm of chromosome 6 encode HLA antigens (Chapter 8). Class I HLA antigens are encoded by three loci: HLA-A, HLA-B, and HLA-C. Class II HLA antigens are encoded by another three loci: HLA-DR, HLA-DP, and HLA-DQ. Serological and molecular testing methods are available to determine an individual's HLA type.[6,7] Although the candidate donors and recipients are tested for HLA-A, -B, -C, -DR, and -DQ antigens, an HLA match requires only that the donor and recipient have compatible HLA-A, HLA-B, and usually HLA-DR antigens. An optimum clinical result occurs when the donor and recipient are matched at the HLA-A, HLA-B, and HLA-DR loci. Even with a complete match, graft rejection and GVHD can occur, indicating the possibility that current testing methods will not recognize antigens that are incompatible or not encoded by MHC. On the other hand, successful transplant can occur even with some degree of HLA mismatch.[8,9]

Approximately 30% of patients requiring allogeneic transplant have a matched sibling donor, ,and another 2–5% have a partially

★ **TABLE 29-2** Type of Transplant, Sources of Stem Cells, and Diseases for Which the Cells Are Used

Type of Transplant	Source of Stem Cells	Diseases
Allogeneic	Bone marrow Peripheral blood Cord blood	Acute leukemias, chronic myelogenous leukemia, non-neoplastic hematologic conditions (Table 29-1), immunologic and inherited diseases
Autologous	Peripheral blood Bone marrow	Hodgkin lymphoma, non-Hodgkin lymphoma, solid organ tumors, plasma cell myeloma
Syngeneic	Peripheral blood Bone marrow	Any disorder treated by allogeneic/autologous transplant except a genetic disease

matched related donor.[10] If family members do not match or are not available, the search for an unrelated HLA-matched donor is made through the National Marrow Donor Program (NMDP) established in 1986 and World Marrow Donor Association (WMDA) founded in 1988.[11,12] This type of transplant is called a *matched unrelated donor (MUD) transplant*. The NMDP and WMDA promote volunteer donation of bone marrow and peripheral blood stem cells and establish and promote the practice of sharing stem cell sources by need rather than the donor's geographic location.[12] Although millions are in the donor pool, the MUD transplant has certain limitations. First, because of HLA polymorphisms, locating HLA-matched donors is difficult. Second, the length of time to find a compatible allograft is often about 4–6 months. Third, the cost of donor search and procurement is high. Fourth, the racial distribution in the NMDP is unbalanced, limiting its usefulness for patients of minority origins, particularly those of African, Mexican, and Asian heritage. However, considerable efforts are being made to recruit minority donors and in 2010, 40% of new donors in the NMDP registry were from diverse ethnic and racial backgrounds. Finally, the chance of graft failure, GVHD, and opportunistic infections with MUD transplant increases.

The initial donor selection for an allogeneic transplant is based on HLA compatibility with the recipient. ABO blood group antigens of the donor and recipient do not need to be matched to accomplish successful transplantation because these antigens are not expressed on HSCs and recipients will become the blood type of the donor or of a mixed chimera of donor and recipient blood types. Although >30% of SCT recipients receive HSCs from ABO-incompatible donors, there are potential adverse consequences, including delayed red blood cell recovery or pure red cell aplasia (recipient's antibodies directed against donor ABO blood type) or delayed hemolysis (from donor B lymphocytes producing ABO antibodies against residual recipient red blood cells [RBCs]).

Stem cell donors are tested for infectious diseases required by the Food and Drug Administration (FDA) for volunteer blood donors. Most transplant centers absolutely exclude donors only if they are found to have serologic evidence of HIV infection.

Before receiving the allogeneic HSC infusion, the recipient is treated with conditioning chemotherapy and/or total body irradiation.[13] Depending on the underlying disease, this regimen can serve two purposes. First, it provides an antitumor effect if the transplant is being done as part of a treatment regimen for a malignant disease. Second, it is immunosuppressive to enable the recipient to better tolerate the donor cells. The goal of giving high-dose chemotherapy/radiotherapy is to destroy all malignant cells, but the therapy is also toxic to normal bone marrow cells. The patient's marrow is then rescued with infused donor stem cells. As in solid organ transplantation, recipients must also be given additional immunosuppressive therapy such as cyclosporine or tacrolimus to minimize graft rejection and GVHD.

Syngeneic transplantation is the use of bone marrow or PBSCs from an identical twin. This type of transplant is rare but could be used for any disorder treated by allogeneic or autologous transplant except genetic diseases that affect both twins. Syngeneic transplants should have no risk of rejection or GVHD, and no special immunosuppressive drugs need be given.

Autologous Stem Cell Transplantation

Autologous stem cell transplantation infuses the patient's own bone marrow or PBSCs. These stem cells are collected prior to intensive or myeloablative chemotherapy and/or radiotherapy, which seek to eliminate the malignant cells. After therapy, the collected stem cells are infused into the patient to re-establish hematopoiesis. Autologous transplantation is usually indicated when the patient's stem cells are not affected by the disease and the underlying disease is potentially responsive to high-dose chemotherapy and/or radiotherapy. This type of transplant is commonly utilized for Hodgkin lymphoma; non-Hodgkin lymphoma; plasma cell myeloma; solid organ tumors including breast, ovarian, and testicular cancers; and pediatric neoplasms such as neuroblastoma and Wilms' tumor (Table 29-2).

Autologous PBSC transplant can be performed if the disease involves the patient's own marrow. However, in this case, a number of other factors should be evaluated: age, underlying disease, degree of marrow involvement, response to previous chemotherapy, and donor availability. The use of autologous stem cells for transplant in a subset of acute leukemias, myeloproliferative disorders, and myelodysplastic syndrome (MDS) has been attempted but without great success.[14–16]

Autologous stem cell transplantation has both advantages and disadvantages. One important advantage is that it is not necessary to identify HLA-matched donors. Graft rejection and GVHD do not occur, so immunosuppression is not used. Peritransplant mortality is low, and older patients tolerate the procedure relatively well. Some disadvantages of autologous transplant include the possibility of neoplastic cells in the stem cell product that could cause disease recurrence, difficulty in obtaining adequate stem cells if the patient has received extensive prior therapy, and the **graft-versus-leukemia (GVL)** effect that is sometimes seen with allogeneic transplant is not possible. GVL effect is a favorable effect seen when immunocompetent donor T cells present in the allograft destroy the recipient's leukemic cells. The significance of GVL is described later in the chapter.

☑ CHECKPOINT 29-1

A physician is evaluating a 28-year-old patient with a history of acute lymphoblastic leukemia for PBSC transplantation. The laboratory professional found circulating leukemic blasts in the peripheral blood. Is this patient a candidate for an autologous PBSC transplant?

Umbilical Cord Stem Cell Transplantation

Umbilical cord blood contains sufficient numbers of HSCs to provide short-term and long-term engraftment in related and unrelated recipients.[17–19] The first cord blood transplantation was performed in a 5-year-old boy with Fanconi's anemia in 1988.[20] Clinical data indicate that cord blood from siblings and unrelated donors can be used to reconstitute hematopoiesis in patients with malignant and nonmalignant disorders.[17–19,21] As a result of the early successes with cord blood transplantation from sibling donors, pilot programs for banking unrelated donor cord blood

★ **TABLE 29-3** Advantages and Disadvantages of Using Cord Blood Stem Cells versus Marrow Stem Cells from Donors

Advantages	Disadvantages
Cord blood is abundantly available.	Total number of stem cells is generally inadequate for most adult transplant patients.
Cord blood stem cells can be collected without risk to mother or infant.	GVL effect can be reduced.
Cord blood can be frozen and made readily available on demand.	Only one unit is available for each transplant procedure.
Ethnic balance of a given population of donors can be maintained.	
The risk of latent viral contamination (CMV, EBV) is low.	
Cord blood does not contain mature T cells and therefore is likely to be less immunogenic and will reduce the incidence of GVHD.	

CMV = cytomegalovirus; EBV = Epstein-Barr virus; GVHD = grat-versus-host disease; GVL = graft-versus-leukemia

were initiated in various countries around the world. Cord blood contains sufficient numbers of HSCs for engraftment in most recipients weighing <40 kg. However, cord blood transplants have been attempted with some success in patients weighing >40 kg. To obtain an adequate HSC dose, the transplantation of two partially HLA-matched cord blood units has been used with success in some adults.[22] See Table 29-3 ★ for the advantages and disadvantages of using cord blood over marrow.

CASE STUDY *(continued from page 558)*

Brandon had a bone marrow examination, and 6% blasts were present. He has four siblings.

1. Is Brandon a candidate for a stem cell transplant?
2. If yes, what form of transplant is required?
3. What testing should be done on Brandon to proceed with the transplant?

☑ CHECKPOINT 29-2

What would be the best form of transplant for a patient with CML who needs an SCT, and what antigen type needs to be matched?

COLLECTION AND PROCESSING OF HEMATOPOIETIC STEM CELLS

Collecting and processing HSCs requires a team of clinical laboratory professionals as well as physicians.

Bone Marrow

Collection of bone marrow stem cells (allogeneic or autologous) is a surgical procedure performed in the operating room using general or local anesthesia. The marrow is taken from the posterior iliac crest with harvest needles and syringes. Approximately 10 to 15 mL of marrow per kilogram of recipient body weight is harvested to provide an adequate number of MNCs. When the marrow is harvested, it contains a mixture of red blood cells, white blood cells, MNCs, platelets, plasma, and fat particles. If a marrow recipient is ABO compatible with the donor, the marrow can be infused immediately after filtering the fat and small bone particles. If the donor and recipient are ABO incompatible, erythrocytes and/or plasma can be removed to avoid adverse reactions by the recipient upon administration. In some cases, the marrow can be further processed to remove a mononuclear cell layer that can be frozen and stored. Stem cell collection by this method is rarely used now and is being replaced by peripheral stem cell collection (see "Peripheral Blood").

Peripheral Blood

Because stem cells are rare in the peripheral blood (<0.01%), various regimens have been tried to mobilize stem cells in the marrow to enter the peripheral blood and increase the number of circulating MNCs. Transplanting more stem cells means more rapid hematopoietic recovery.[23] For autologous PBSC collection, patients can receive cytotoxic chemotherapy (e.g., Cytoxan), hematopoietic cytokines (e.g., granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF]), or a combination of chemotherapy and cytokines to mobilize stem cells.

After cytotoxic and cytokine therapy, the stem cells in the marrow are mobilized to the peripheral blood where they can be collected by **apheresis**, an automated procedure that uses a blood cell separator. Whole blood is withdrawn from the donor and anticoagulated. The components are separated using centrifugation or filtration, and the desired component is collected (in this case, mononuclear cells that contain HSCs). The remaining components are combined and infused back into the donor.

For allogeneic PBSC collection, hematopoietic cytokines (G-CSF or GM-CSF) are given to normal, healthy donors to mobilize stem cells, and then the MNCs are collected by apheresis.

The optimum timing for collection of PBSCs after cytotoxic and growth factor therapy is sometimes unpredictable, and the time of harvest varies depending on the mobilization protocol. Some centers determine the timing of PBSC collection based on a preapheresis peripheral blood CD34 count.[24]

CASE STUDY *(continued from page 561)*

An HLA-matched sibling has agreed to be a donor for Brandon.

4. Should the source of stem cells be peripheral blood or bone marrow? Why?

Umbilical Cord Blood (UCB)

If UCB is to be collected as a source of stem cells, the mother must sign an informed consent, and a complete medical history of the parents is obtained. Cord blood is usually collected from a delivered placenta or sometimes from an undelivered placenta (after the baby is delivered and the cord is being clamped). A large-bore needle is inserted into the umbilical vein to collect blood in a sterile collection bag containing anticoagulant. Several methods are used to separate MNCs from the whole cord blood: Ficoll-Hypaque, modified Ficoll-Hypaque gradient method, and addition of hydroxyethyl starch (HES).[25,26] The cord blood is not usually processed if certain conditions are present (Table 29-4 ★).

Purging

Purging is a technique to reduce possible tumor cell contamination or in the setting of allogeneic transplant, to reduce the number of T lymphocytes present in the product. Purging is performed on stem cell products that have been collected by apheresis. Depending on the source of stem cells and clinical conditions in which the cells are used, various purging techniques (mechanical, immunological, and pharmacological) are available.[27]

In an allogeneic transplantation, GVHD, an immunologic response that results from the presence of the donor's T lymphocytes that can interact with the host's cells, is a potential complication, but purging the donor T lymphocytes from the allograft can prevent or reduce its severity. However, some of these donor T cells seem to be necessary for marrow engraftment and for achievement of the GVL effect. A favorable response, GVL is usually seen because the T cells present in the graft can have an active role in destroying the residual leukemic cells of the recipient. In support of this possibility, patients receiving T-cell–depleted grafts are known to be at higher risk for disease relapse than patients receiving unmanipulated grafts for the same disease.

★ **TABLE 29-4** Conditions in Which Stem Cells Are Not Usually Collected and/or Processed from the Cord Blood

Inadequate volume collected from individual placenta (<40 mL)
Preterm delivery (<36 weeks)
Fever higher than 100° F (38°C) in mother
Premature rupture of membranes
Meconium staining of fluid
Family history of inherited diseases
Congenital anomalies in infant

Cryopreservation and Storage of Hematopoietic Stem Cells

HSCs from bone marrow, PBSCs, or cord blood can be effectively **cryopreserved** (stored at very low temperatures) to maintain cell viability for months or years. When the stem cells are frozen or thawed, cell injury or loss of stem cell viability results from intracellular crystal formation and cellular dehydration.

To achieve the optimum cryopreservation without compromising the cell viability, a cryoprotectant agent is used.[27] The most common cryoprotectants are either 10% dimethylsulfoxide (DMSO) or 5% DMSO with 6% HES. The DMSO diffuses rapidly into the cells and increases intracellular solute concentration, thus decreasing intracellular ice crystal formation and preventing cell lysis.

After optimizing the concentration of the stem cells in the presence of cryoprotectant, tissue culture media and autologous plasma are frozen in many small plastic bags. The freezing rate seems to be an important factor for viable cell recovery. The freezing is usually performed in the chamber of a programmable device into which liquid nitrogen is pumped to maintain the desired rate. When the freezing program has been completed, the bags are removed from the chamber and placed in a liquid nitrogen storage unit in either liquid or vapor phase at −196° C.

Infusion of Hematopoietic Stem Cells

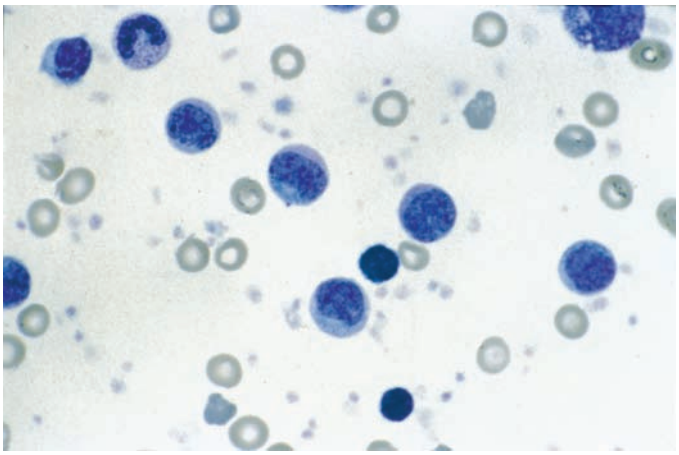
The patient to be infused is premedicated with antihistamine and antiemetic (sometimes diuretics and/or steroids). The frozen bags are immediately thawed in a 37°C water bath just before the infusion. The infusion is carried out as quickly as possible to dilute the cell suspension with the patient's blood volume because DMSO in liquid phase can be toxic to stem cells. The most common adverse reactions associated with cryopreserved stem cells are chills, nausea, vomiting, fever, allergic reactions, transient cough, and shortness of breath. Adverse events from HSC infusion can be related to DMSO-induced histamine release; therefore, some centers thaw and dilute or wash the cell suspension to remove the DMSO, but cell loss and clumping can be a problem with this approach.

QUANTITATION OF HEMATOPOIETIC STEM CELLS

The number of stem cells or CD34+ cells that can be obtained varies greatly from donor to donor. For PBSC, one or more apheresis procedures are needed to acquire the appropriate dose for engraftment and hematopoietic recovery. Because the engraftment outcome depends on the number of stem cells present in the product, the HSCs (from bone marrow, PBSCs, or cord blood) are quantitated by one of the three methods described next.

Determination of Mononuclear Cell Count

Stem cells are mononuclear cells (as are immature myeloid cells, lymphocytes, and monocytes); thus, counting the MNCs provides a very indirect estimate of the number of stem cells present in the sample (Figure 29-1 ■). The volume of cells necessary to achieve a satisfactory dose for successful engraftment can be determined from the MNC count. The dose is usually specified as the number



■ **FIGURE 29-1** Wright's stain slide prepared for the manual differential count from the stem cell product showing myeloid precursors and mononuclear cells (peripheral blood, Wright's stain, 1000× magnification).

of MNCs per kilogram of the recipient's body weight. Laboratories use automated cell counters or hemacytometers to count the MNCs.

CD34 Enumeration by Flow Cytometry

Enumerating cells that express CD34 antigen (stem cells and progenitor cells) by immunophenotyping has become a routine practice. When bound to fluorescent dyes, anti-CD34 antibodies offer a rapid method of enumerating these cells using a flow cytometer. Flow cytometric enumeration of CD34+ cells has been shown to be the most useful indicator of the hematopoietic reconstitutive capacity of SCT.

The major difficulty with the analysis of CD34+ cells is the low number of cells present in the specimen. The method's sensitivity is 1 in 10,000. Procedures to measure CD34+ cells vary widely. Optimally, 100 CD34+ cells and 75,000 CD34+ (leukocytes) events should be acquired to optimize the accuracy of analysis.[28,29] The CD34+ count is calculated in conjunction with the total leukocyte counts.[29,30] CD34+ counts can be performed on peripheral blood and the stem cell product. Results should be reported as soon as possible because the decision to collect more stem cells depends on CD34+ yield. Some centers determine the timing of PBSC collection based on a peripheral blood CD34+ count.[24,31] If the CD34+ cells are lower than required, the decision can be made to perform (or postpone) more apheresis or to mobilize the stem cells in a different manner.

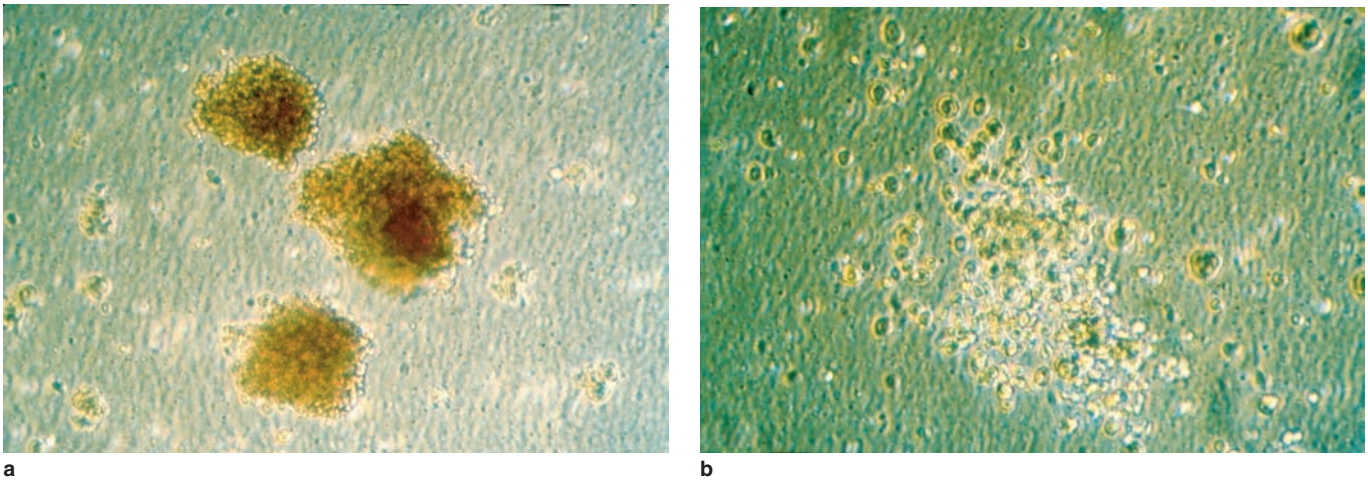
Cell Culture for Colony Forming Units

Flow cytometry analysis identifies cells only by their antigenic markers and thus cannot identify cells that are **clonogenic** (capable of proliferating and forming colonies of cells when grown in vitro); only culture techniques can demonstrate clonogenicity. An in vitro clonogenic assay is a culture system containing stem cells and various growth factors. The culture is incubated for 14–15 days on a semisolid medium (agar or methylcellulose). The colonies generated from committed myeloid and erythroid hematopoietic progenitors can be differentiated and counted (Figure 29-2 ■).

The advantage of the clonogenic assay is that this system tests the capacity of progenitor cells to divide and can predict the engraftment potential of the stem cell product.[32,33] The 2-week incubation period needed in this method is a distinct disadvantage when data are needed for immediate clinical decisions. The results of clonogenic assay systems from different institutions vary greatly because of variability in reagent lots and the level of staff expertise. Commercial kits are now available to aid in the standardization of clonogenic assays.

☑ CHECKPOINT 29-3

You receive a peripheral blood specimen in the hematology laboratory with a request for a mononuclear cell count and analysis of CD34+ cells. Without any other information, why should you consider this a STAT request?



a **b**

■ **FIGURE 29-2** Cell culture colonies. **(a)** Erythroid colonies. **(b)** Myeloid colonies (original magnification ×100, no stain on semisolid medium).

COLLECTION TARGET FOR STEM CELLS

The recipient's body weight determines the required dose of stem cells. The exact number of HSCs needed to ensure engraftment is not known, but cell doses of 2.5–5.0 $\times$ 10^6 CD34+ cells/kg of recipient weight are desirable; earlier engraftment is observed with even higher doses.[34,35] For a transplant using umbilical cord blood, >3.0 $\times$ 10^7 of total nucleated cells per kg of recipient weight is desirable.

CASE STUDY (continued from page 562)

PBSCs were collected by apheresis from the sibling. Enumeration of CD34 count indicates that the total CD34+ cells collected are 6 $\times$ 10^6/kg.

5. Is this an adequate dose for SCT?

HEMATOPOIETIC ENGRAFTMENT

The **conditioning regimen** given to patients for SCT causes severe myelosuppression and can affect T- and B-cell immunologic responses (usually in an allogeneic setting). After the conditioning regimen, the HSCs are infused to restore the marrow function. A period of pancytopenia typically follows during which the patient is prone to develop infections and bleeding complications. To reduce the period of pancytopenia, which can last from a few days to 2–4 weeks, growth factors such as G-CSF or GM-CSF can be given.[35,36] Recovery of myeloid cells usually occurs first and is followed by platelet and red cell recovery.

Evidence of Initial Engraftment

Evidence of hematopoietic engraftment exists when the absolute neutrophil count is >500/mcL (μL) and the platelet count is >20,000 mcL with no need for platelet transfusions. Compared with bone marrow transplants, the time a recipient is pancytopenic following a PBSC transplant is somewhat shorter. After cord blood transplants, neutrophil recovery can take 5–6 weeks, and the platelets usually engraft late (median time 80 days).[25]

Evidence of Long-Term Engraftment

When all hematopoietic cells in an SCT recipient are derived from an allogeneic donor, the condition is referred to as full or complete **chimerism** (the presence of only donor hematopoietic cells in a recipient). When a recipient's hematopoietic cells persist together with donor cells after SCT, the condition is referred to as *partial* or *mixed chimerism*.

To evaluate the long-term engraftment of HSCs, various laboratory methods have evolved.[37] Detection of red cell antigens was among the first tests used to evaluate donor cell engraftment. For example, if the recipient's blood group is O and the donor's blood group is A, the recipient can type as group A after hematopoietic engraftment with complete chimerism. However, because of the long life span of red cells and multiple blood transfusions during transplantation, the red cell antigens are no longer used for chimerism studies.

Several molecular techniques can be used to evaluate chimerism in transplant patients. If the donor and recipient are of opposite sex, fluorescent in situ hybridization (FISH) analysis of X and Y chromosomes can be used to monitor engraftment (Chapter 42).[38,39]

Currently, the most widely used methodology for monitoring chimerism is by polymerase chain reaction amplification of short tandem repeat (STR) loci followed by capillary electrophoresis to distinguish the amplicons (Chapter 42).[38–40] STRs are short sequences of DNA repeated numerous times. Individuals can have unique numbers of copies of the repeat element, and this individual copy number is referred to as a *polymorphism*. SCT generally involves donation between closely related individuals, so multiple STR loci must be analyzed to differentiate donor from recipient cells. Loci that discriminate between individuals are said to be *informative*; loci that do not discriminate are said to be *not-informative*.

In summary, the clinical applications of chimerism tests in marrow transplantation are to document donor cell engraftment, evaluate the persistence of donor cells, and assess the recurrence of disease.

☑ CHECKPOINT 29-4

A physician wants to evaluate the engraftment on a male patient who received SCT from his brother 4 months ago. What laboratory tests should be performed to make this assessment?

CASE STUDY

Stem cells were collected and frozen for Brandon.

6. Does Brandon need to undergo any form of therapy before the transplant?

ROLE OF THE CLINICAL LABORATORY PROFESSIONAL IN STEM CELL TRANSPLANTATION

When the decision to undertake an SCT has been made, collecting and processing stem cells involves a dedicated clinical laboratory staff who work as a team (Figure 29-3 ■). The SCT process involves the clinical laboratory professionals working in the departments of hematology, apheresis, blood bank, microbiology, flow cytometry,

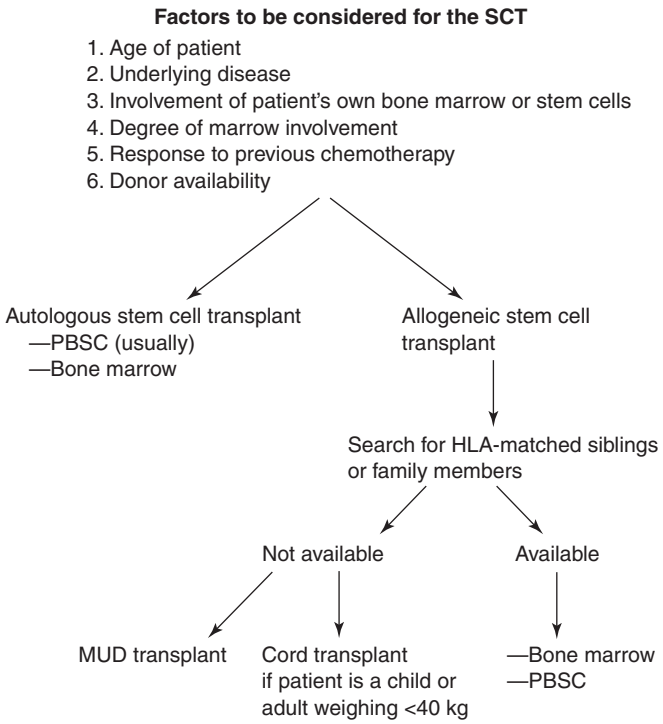


FIGURE 29-3 Decision-making process in stem cell transplantation. The patient is usually evaluated for SCT at the time the primary diagnosis is made. For allogeneic SCT, a donor is selected based on HLA compatibility. If family members do not match or are not available, the search for an unrelated HLA-matched donor is made through the NMDP. If the patient is a child or small adult (<40 kg), the cord blood registry/banks is/are also options. In general, if the disease involves the patient's own stem cells, allogeneic transplant is a valid option. Autologous transplant is planned when the disease has not affected the patient's own stem cells. On the other hand, autologous PBSC transplant can be performed even if the disease involves the patient's marrow, but a number of other factors should be evaluated as shown in the algorithm.

PBSC = peripheral blood stem cell; MUD = matched unrelated donor

molecular/HLA, cytogenetics, and bone marrow transplant laboratories (Figure 29-4 ■).

Consider a patient for whom the clinical decision for autologous SCT has been made. This patient will receive cytotoxic chemotherapy, G-CSF, or both. For PBSC collection, the patient will undergo apheresis. (In an allogeneic transplant setting, both the donor and recipient cells are typed for HLA antigens in the molecular/HLA laboratory before collecting stem cells.) The apheresis staff will spend 4–6 hours with the patient to get a good yield of MNCs. After collecting the product, the sample will be sent to the hematology and flow cytometry labs where WBCs/MNCs and CD34+ counts will be performed on a sample of the stem cell product collected by apheresis.

The bone marrow transplant laboratory staff will process and cryopreserve the stem cells for future use. The cells' viability will be checked, samples for bacterial cultures taken, and the cell culture set up for enumeration of CFUs. On the day of transplant, the clinical laboratory staff (usually from the bone marrow laboratory) will thaw the stem cell units for the infusion.

After the transplant, hematology will evaluate initial (short-term) engraftment with a complete blood count. For evaluation of long-term engraftment, the sample will be sent to the cytogenetic and molecular/HLA laboratories to perform chimerism studies. Throughout the course of the transplant, the blood bank personnel will provide special blood components required during the transplantation process.

GRAFT-VERSUS-HOST DISEASE AND GRAFT-VERSUS-LEUKEMIA EFFECT

The exact mechanism for GVHD is not clearly known. However, its suggested pathogenesis is that immunocompetent donor T lymphocytes recognize HLA antigens on the host (recipient) cells as foreign and initiate secondary inflammatory injury mediated by inflammatory cytokines (interleukin-1, tumor necrosis factor [TNF]). This leads to tissue injury.[41,42]

Three factors necessary to set the stage for GVHD are the presence of immunocompetent donor T lymphocytes, HLA alloantigens, and an immunosuppressed host. GVHD is more frequent and severe in recipients of partially HLA-matched sibling allografts or in grafts from unrelated donors.[8] However, GVHD also occurs after HLA-identical sibling donor allografts, indicating the role of other HLA minor antigens or factors that are not usually tested for at the time of transplant.

Overall, the risk of acute GVHD (for the degree of HLA mismatch) is less in recipients of cord SCT than in recipients of a marrow transplant, perhaps because of the immature nature of cord blood immune cells. GVHD can be prevented or its severity reduced by decreasing the number of donor T lymphocytes from the graft (purging) and/or administering medications such as cyclosporine A, methotrexate, steroids, cyclophosphamide, antithymocyte globulin, and tacrolimus.[43]

Recipients of T-cell–depleted marrow experience GVHD less frequently but have a higher incidence of disease relapse and higher graft failure than patients treated with unmanipulated marrow for the same disease. T lymphocytes are known to be essential for marrow engraftment and for achieving desirable GVL effect, which is seen when immunocompetent donor T cells present in the allograft destroy the recipient's leukemic cells.[44] For example, in allograft recipients with CML who relapse after allogeneic SCT, infusion of small numbers of lymphocytes collected by apheresis from the original graft donor (donor lymphocyte infusion or DLI)[45] can induce a potent GVL effect and re-establish complete remission in most patients.

The critical issue of how many T lymphocytes or which subpopulations of T lymphocytes need to be removed from the allograft to avoid GVHD but allow marrow engraftment and the GVL effect is unknown. The T-cell concentration and subsets required to achieve long-term engraftment, control of GVHD, and beneficial GVL effect also depend on the disease and the degree of HLA disparity.

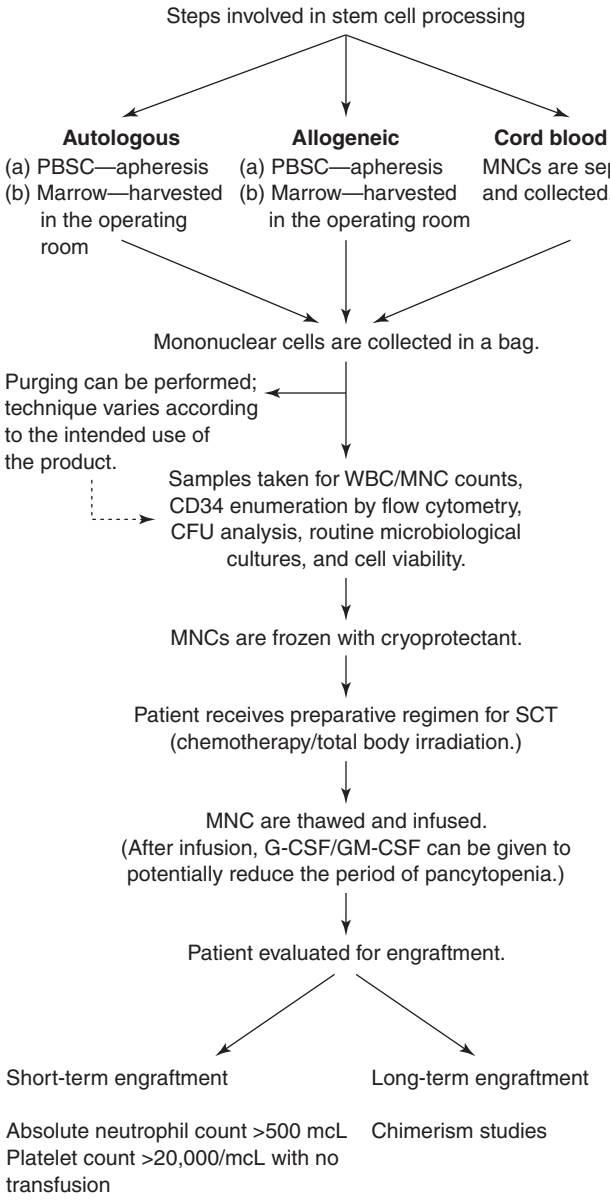


FIGURE 29-4 Steps involved in stem cell transplantation from the time the patient is diagnosed with the disease to the final infusion of stem cell product. For autologous transplant, the stem cells are usually collected from peripheral blood or are sometimes harvested from the bone marrow. If the stem cells are to be collected from the peripheral blood, the patient can receive cytotoxic chemotherapy or growth factors, or both to mobilize stem cells into the blood; the stem cells are then collected by apheresis. For an allogeneic transplant, an HLA-matched family member donor or an HLA-matched donor from the NMDP is considered. During a surgical procedure, the stem cells are collected from the iliac crests. In an allogeneic setting, stem cells can also be collected by apheresis from peripheral blood after mobilizing the donor stem cells with growth factors. Whatever the method used, after collecting the required number of stem cells from peripheral blood, bone marrow, or cord blood, samples are taken for appropriate tests, and stem cells are cryopreserved for later infusion. Before the transplant, the patient receives a high dose of chemotherapy and/or radiotherapy to destroy the tumor cells. This therapy can be toxic to normal organs, including bone marrow. The marrow is rescued by transplanting frozen stem cells that have been reserved for a particular patient.

COMPLICATIONS ASSOCIATED WITH STEM CELL TRANSPLANTATION

Complications with SCT can be categorized as early and late. Complications that occur during the first 100 days after transplantation are considered early complications; those that occur from a few months (>3 months) to several years after transplantation are considered late complications.

Early Complications

Early complications include graft failure, GVHD, peritransplant infections, and recurrence of malignant disease.

Graft Failure

Primary graft failure is the failure to establish hematologic engraftment and can be defined as failure to attain an absolute neutrophil count >500 mcL by 28 days after transplant. *Secondary graft failure* is the loss of an established graft and is defined as the redevelopment of pancytopenia at any time after primary engraftment. These complications can be seen in the allogeneic transplant setting but rarely in the autologous setting. See Table 29-5 ★ for causes of graft failure.

Graft-versus-Host Disease (GVHD)

Classification of GVHD can be either acute or chronic. *Acute GVHD* generally occurs in the first 3 months after allogeneic SCT and usually involves the recipient's skin (maculopapular dermatitis), liver (elevation of bilirubin, abnormal liver function tests), and gastrointestinal tract (nausea, vomiting, diarrhea). *Chronic GVHD* is defined as symptoms appearing 80–100 days after transplantation.

Peritransplant Infections

During the peritransplant period when the patient has been immunosuppressed and has a low neutrophil count, the risk of opportunistic infection is very high. These infections during the first 3 months post-transplant can be serious but rarely life threatening. Infections can be bacterial, protozoal, fungal, and/or viral. Reactivated cytomegalovirus (CMV), a member of the herpes virus family, and herpes simplex are the most common. In the United States, the range of CMV prevalence in the population is 50–85%. SCT patients are particularly vulnerable to CMV infection, which can be primary, but superinfection with a second strain or a reactivation of latent disease also can occur. The complications include pneumonitis, gastroenteritis, and retinitis. Fatal CMV pneumonia occurs in 10–15% of patients.

The relative risk for CMV infection among SCT patients depends on the serological status of both the donor and the recipient.[46] A seronegative recipient of a seropositive transplant is at significant risk of primary transfusion-transmitted CMV infection. Seropositive recipients of seropositive transplants can develop reactivation or superinfection of CMV.

A donor for a CMV-negative patient should be an HLA-compatible person who is seronegative for CMV. SCT patients who are at significant risk for acquiring transfusion-transmitted CMV also should receive cellular components that carry a reduced risk of CMV. It has been shown that CMV resides in leukocytes, and studies have indicated that leukocyte-reduced cellular blood components can prevent CMV infection.[47–49] However, the equivalent efficacy of leukocyte-reduced and CMV-seronegative cellular components in preventing transfusion-associated (TA) CMV has not yet been established. Most transplant physicians believe that if a CMV-seronegative recipient receives stem cells from a CMV-seronegative donor, all subsequent blood components received should be CMV-seronegative. If the CMV-negative blood component is not available, transfusing a leukocyte-reduced filtered component can effectively prevent the transmission of CMV disease.

★ **TABLE 29-5** Causes of Graft Failure

Inadequate number of stem cells
HLA disparity between donor and recipient
Significant T-cell depletion of the allograft
Decreased degree of immunosuppression

Recurrence of Malignant Disease

Patients who undergo autologous transplantation are more at risk of dying from disease recurrence than from transplant-related complications. After an autologous transplant, recurrence of the original disease can occur because of incomplete eradication of malignant cells in the patient or the presence of residual neoplastic cells in the autograft that have not been adequately eliminated by purging techniques. Patients who receive an allogeneic rather than an autologous transplant for hematologic malignancies have a lower relapse rate; however, the risk of leukemic relapse varies widely from 5–70%. This variability depends on the diagnosis and stage of the disease, the degree of immunosuppression, the degree of match or mismatch of the allograft, and the presence or absence of GVHD.

Other Complications

Symptoms and clinical signs of toxicity related to chemotherapy and radiotherapy—including mucositis, myocarditis, pericarditis, pneumonitis, hemorrhagic cystitis, and adverse drug reactions—can occur after SCT. A serious liver disorder that complicates up to 50% of marrow transplants is called *veno-occlusive disease*. It is characterized by right upper quadrant pain, weight gain, and jaundice and is diagnosed clinically. Other features include ascites, hepatomegaly, hyperbilirubinemia, encephalopathy, and renal or multiorgan failure.

Late Complications

Late complications can be secondary to pretransplantation chemotherapy/radiotherapy, continued effects of acute complications, and/or immunosuppressive states leading to delayed infections. These complications include hypothyroidism, hypogonadism, cataracts, growth retardation in pediatric patients, neuropathies, and sometimes development of post-transplant lymphoproliferative disorders and second malignancies (myelodysplastic syndrome and leukemia).

☑ CHECKPOINT 29-5

A CMV-seronegative patient requires SCT. Two HLA-matched donors are available. Is it important to know the stem cell donor's CMV status? If the stem cell donor is CMV seronegative and the patient requires red cell transfusion during the peritransplant period, what blood components (in terms of CMV status) would you select for this patient?

CASE STUDY *(continued from page 564)*

Brandon received stem cells from his HLA-matched sibling that successfully engrafted. Three months later, he developed diarrhea, skin rash, and jaundice.

7. What could be the possible cause for this?

INCREASED AVAILABILITY AND SUCCESS OF STEM CELL TRANSPLANTATION

About 28,000 autologous and 21,000 allogeneic hematopoietic stem cell transplants are performed every year worldwide,[50] and the number increases by about 10–20% annually. Outcomes are improving with reduced mortality, which can be related to reductions in organ damage, infection, and severe GVHD.[51] According to one study, of patients who survived at least 2 years after autologous HSCT for a hematologic neoplasm, about 69% were alive 10 years after transplantation.[52] These statistics indicate that it is imperative for clinical laboratory professionals to work closely with physicians who perform these procedures and stay abreast of developments in this area.

Gene Therapy

In addition to HSCT, gene therapy is a technology that offers the possibility for a complete cure for genetic disease.[53] A complete discussion of this technology is beyond the scope of this book. Suffice it to say that the goal of gene therapy is to introduce a functional copy of the patient's defective gene into a sufficient number of the appropriate cell types (such as stem cells) and to have sufficient expression of that gene so that its product functions to correct the deficiency. Attempts have been made to use genetically engineered viruses to carry the DNA of interest into host cells. Viral genes required for propagation are replaced with a working copy of the human gene. This form of therapy has been used (with variable success) to treat conditions such as adenosine deaminase deficiency, chronic granulomatous disease, X-linked combined severe immune deficiency, and hemophilia B. For stem cell gene therapy to find more widespread clinical utility, several important related issues—such as the isolation of the appropriate cell population for transduction, efficiency of the transduction process, and safe insertion of the functionally correct gene into the recipient's genome—must be overcome.

Summary

HSCT is a recognized therapeutic modality for leukemias, lymphomas, solid organ tumors, and a variety of metabolic/immunologic disorders. Sources of HSCs are bone marrow, peripheral blood, and umbilical cord blood. For an autologous transplant, the patient's own stem cells are collected, frozen, and used later for hematopoietic reconstitution. For an allogeneic transplant, the donor is selected based on the best HLA match from either family members or an unrelated donor. A team of health care professionals including clinical laboratory professionals is needed to collect, process, and quantitate stem cells before administering them to the patient. For autologous PBSC collection, patients can receive chemotherapy and cytokines to mobilize stem cells. For allogeneic PBSC collection, hematopoietic cytokines can be given to the donor. The number of stem cells collected varies from donor to donor, and engraftment partially depends on the number of stem cells in the product. The number of stem cells in the transplant collection can be determined by quantitating the MNCs, cells, and CFUs. Before transplant, the patient undergoes conditioning chemotherapy/radiotherapy after which the stem cells are infused. Routine blood counts and chimerism studies (in allogeneic SCT only) can be performed to assess engraftment. Complications of SCT include graft failure, GVHD, opportunistic infections, and recurrence of malignant disease.

Review Questions

Level I

1. Which of the following property(ies) can be attributable to stem cells? (Objective 3)

   A. self-renewal

   B. multilineage differentiation

   C. mononuclear morphology

   D. all the above

2. What is (are) the source(s) of hematopoietic stem cells? (Objective 1)

   A. bone marrow

   B. peripheral blood

   C. umbilical cord blood

   D. all of the above

3. What form of transplant can be used for inherited genetic diseases? (Objective 2)
   A. autologous stem cell transplant
   B. allogeneic stem cell transplant
   C. syngeneic stem cell transplant
   D. stored autologous cord blood
4. What is a syngeneic transplant? (Objective 1)
   A. infusion of stem cells from any donor into the recipient
   B. infusion of patient's own stem cells
   C. infusion of stem cells from an identical twin
   D. infusion of stem cells from different species
5. What cells are usually CD34+? (Objective 3)
   A. maturing myeloid cells
   B. stem cells
   C. B lymphocytes
   D. T lymphocytes
6. Which of the following is correct regarding allogeneic stem cell transplant and ABO antigens? (Objective 4)
   A. ABO antigens do not need to be matched.
   B. ABO antigens are strongly expressed on stem cells.
   C. The O group patient should not receive stem cells from A or B blood group donors.
   D. ABO antigens should always be matched.
7. What is the single most important factor to consider in donor selection for an allogeneic transplant? (Objective 4)
   A. HLA compatibility with the recipient
   B. ABO compatibility with the recipient
   C. CMV status of the donor
   D. HLA and ABO compatibility with the recipient
8. Which of the following infections can be serious in the peri-transplant period? (Objective 5)
   A. bacterial
   B. fungal
   C. cytomegalovirus
   D. all of the above
9. Severe GVHD usually is seen in which group of patients? (Objective 6)
   A. syngeneic SCT
   B. autologous SCT
   C. unmatched allogeneic SCT
   D. umbilical cord stem transplant
10. What organ(s)/tissue(s) is(are) usually involved in GVHD? (Objective 6)
    A. skin only
    B. skin, liver, and/or the gastrointestinal tract
    C. liver only
    D. gastrointestinal tract only

Level II

1. What does the term *chimerism* mean? (Objective 6)
   A. recovery of recipient's red cells
   B. absence of malignant cells
   C. presence of donor hematopoietic stem cells in a recipient
   D. recovery of patient's white cells and platelets only
2. What is the most appropriate form of transplant for an adult patient with CML weighing 80 kg? (Objective 7)
   A. autologous SCT
   B. umbilical cord SCT
   C. allogeneic SCT
   D. none of the above; transplant is not indicated in patients with CML
3. A risk for GVHD is carried by a patient who has: (Objective 4)
   A. received allogeneic SCT
   B. received autologous SCT
   C. received both allogeneic and autologous SCT
   D. received autologous SCT for only solid organ tumors
4. What are the three factors necessary to set the stage for GVHD? (Objective 4)
   A. immunocompetent donor T lymphocytes, ABO antigens, and immunosuppressed host
   B. immunosuppressed donor T lymphocytes, HLA alloantigens, and immunosuppressed host
   C. immunocompetent donor B lymphocytes, HLA alloantigens, and immunosuppressed host
   D. immunocompetent donor T lymphocytes, HLA alloantigens, and immunosuppressed host
5. What procedure usually is used to quantify HSCs? (Objective 3)
   A. lymphocyte count
   B. CD4+ count by flow cytometry
   C. monocyte count by automated cell counter
   D. CD34+ count by flow cytometry

6. Patients who receive this type of stem cell transplant are more at risk for disease recurrence than of transplant-related complications. (Objective 4)

   A. allogenic

   B. syngeneic

   C. cord blood

   D. autologous

7. This test is appropriate to determine long-term engraftment of HSCs only when donor and recipient are of the opposite sex. (Objective 6)

   A. detection of RBC antigens

   B. Fluorescent in situ hybridization with sex chromosomes

   C. CD34+ cell enumeration by flow cytometry

   D. platelet and neutrophil counts

8. GVL effect is seen after SCT in patients who have received: (Objective 4)

   A. autologous transplant

   B. allogeneic transplant

   C. either autologous or allogeneic transplant

   D. umbilical cord transplant

9. For autologous peripheral SCT, the patient usually undergoes therapy in which sequence? (Objective 5)

   A. myeloablative therapy, apheresis, and then G-CSF

   B. G-CSF followed by myeloablative therapy and then apheresis

   C. apheresis to collect CD34+ cells, myeloablative therapy, and then infusion of collected CD34+ cells.

   D. myeloablative therapy and then apheresis to collect CD34+ cells

10. What is the sequence in which successful engraftment occurs after SCT? (Objective 6)

    A. Neutrophils are increased first followed by an increase in platelets.

    B. Platelets are increased first followed by an increase in neutrophils.

    C. Red cells are increased first followed by an increase in platelets.

    D. Red cells and platelets are increased first followed by an increase in neutrophils.

Companion Resources

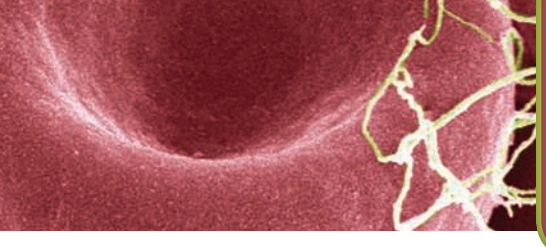
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Jacobson LO, Marks EK, Robson MJ et al. Effect of spleen protection on mortality following x-irradiation. *J Lab Clin Med*. 1949;34:1538–43.
2. Lorenz E, Uphoff D, Reid TR et al. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst*. 1951;12:197–201.
3. Wilson A, Trumpp A. Bone marrow hematopoietic stem cell niches. *Nat Rev Immunol*. 2006;6(2):93–106
4. Speck B, Bortin MM, Champlin R et al. Allogeneic bone-marrow transplantation for chronic myelogenous leukaemia. *Lancet*. 1984;1:665–68.
5. Copeland EA. Hematopoietic stem cell transplantation. *N Engl J Med*. 2006;354(17):1813–826
6. Begovich AB, Erlich HA. HLA typing for bone marrow transplantation: new polymerase chain reaction-based methods. *JAMA*. 1995;273:586–91.
7. Beatty PG. The immunogenetics of bone marrow transplantation. *Transfus Med Rev*. 1994;8:45–58.
8. Beatty PG, Clift RA, Mickelson EM et al. Marrow transplantation from related donors other than HLA-identical siblings. *N Engl J Med*. 1985;313:765–71.
9. Vogelsang GB, Hess AD, Santos GW. Acute graft-versus-host disease: clinical characteristics in the cyclosporine era. *Medicine*. 1988;67:163–74.
10. Kernan NA, Bartsch G, Ash RC et al. Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. *N Engl J Med*. 1993;328:593–602.
11. National Marrow Donor Program Survey. www.marrow.org; accessed December 13, 2012.
12. Perumbeti A, Sacher RA, Besa EC. Hematopoietic stem cell transplantation. Medscape Reference. 2012 (retrieved from emedicine.medscape.com/article/208954-overview; accessed November 8, 2013).
13. Heslop HE. Overview of hematopoietic stem cell transplantation. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Saunders; 2012:1542–545.
14. Stein AS, Forman SJ. Autologous hematopoietic stem cell transplantation for acute myeloid leukemia. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic Stem Cell Transplantation*, 2nd ed. Boston: Blackwell Science; 1999:963–77.
15. Armitage JO. Bone marrow transplantation. *N Engl J Med*. 1994;330:827–38.
16. Blaise D, Gaspard MH, Stoppa AM et al. Allogeneic or autologous bone marrow transplantation for acute lymphoblastic leukemia in first complete remission. *Bone Marrow Transplant*. 1990;5(1):7–12.
17. Ballen KK. New trends in umbilical cord blood transplantation. *Blood*. 2005;105(10):3786–92.
18. Wagner JE, Rosenthal J, Sweetman R et al. Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. *Blood*. 1996;88:795–802.
19. Gluckman E, Rocha V, Boyer-Chammard A et al. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant

Group and the European Blood and Marrow Transplantation Group. *N Engl J Med*. 1997;337:373–81.
20. Gluckman E, Broxmeyer HE, Auerbach AD et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med*. 1989;321:1174–178.
21. Rubinstein P, Carrier C, Scaradavou A et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med*. 1998;339:1565–577.
22. Scaradavou A, Brunstein CG, Eapen M et. al. Double unit grafts successfully extend the application of umbilical cord blood transplantation in adults with acute leukemia. *Blood*. 2013;121:752–58.
23. Papayannopoulou T. Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. *Blood*. 2004;103(5):1580–585.
24. Schots R, Van Riet I, Damiaens S et al. The absolute number of circulating CD34+ cells predicts the number of hematopoietic stem cells that can be collected by apheresis. *Bone Marrow Transplant*. 1996;17:509–15.
25. Rubinstein P, Dobrila L, Rosenfield RE et al. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc Natl Acad Sci U S A*. 1995;92(10):119–22.
26. Harris DT, Schumacher MJ, Rychlik S et al. Collection, separation, and cryopreservation of umbilical cord blood for use in transplantation. *Bone Marrow Transplant*. 1994;13:135–43.
27. Wissel ME, Lasky LC. Progenitor processing and cryopreservation. In: Brecher ME, Lasky LC, Sacher RA et al., eds. *Hematopoietic Progenitor Cells: Processing, Standards, and Practice*. Bethesda, MD: AABB; 1995:109–24.
28. Sims LC, Brecher ME, Gertis K et al. Enumeration of CD34-positive stem cells: evaluation and comparison of three methods. *J Hematother*. 1997;6:213–26.
29. Sutherland DR, Anderson L, Keeney M et al. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. *J Hematother*. 1996;5:213–26.
30. Keeney M, Chin-Yee I, Weir K et al. Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. International Society of Hematotherapy and Graft Engineering. *Cytometry*. 1998;34:61–70.
31. Gambell P, Herbert K, Dickinson M et al. Peripheral blood CD34+ cell enumeration as a predictor of apheresis yield: an analysis of >1,000 collections. *Biol Blood Marrow Transplant*. 2012;18(5):763–72.
32. Lasky LC, Johnson NL. Quality assurance in marrow processing. In: Areman EM, Deeg HJ, Sacher RA, eds. *Bone Marrow and Stem Cell Processing: A Manual of Current Techniques*. Philadelphia: FA Davis; 1992:386–443.
33. Douay L, Gorin NC, Mary JY et al. Recovery of CFU-GM from cryopreserved marrow and in vivo evaluation after autologous bone marrow transplantation are predictive of engraftment. *Exp Hematol*. 1986;14:358–65.
34. Bensinger W, Appelbaum F, Rowley S et al. Factors that influence collection and engraftment of autologous peripheral-blood stem cells. *J Clin Oncol*. 1995;13:2547–555.
35. Haas R, Mohle R, Fruhauf S et al. Patient characteristics associated with successful mobilizing and autografting of peripheral blood progenitor cells in malignant lymphoma. *Blood*. 1994;83:3787–794.
36. Siena S, Schiavo R, Pedrazzoli P et al. Therapeutic relevance of CD34 cell dose in blood cell transplantation for cancer therapy. *J Clin Oncol*. 2000;18(6):1360–377.
37. Bryant E, Martin PJ. Documentation of engraftment and characterization of chimerism following hematopoietic cell transplantation. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic Stem Cell Transplantation*, 2nd ed. Boston: Blackwell Science; 1999:197–206.
38. Murphey K. Chimerism analysis following hematopoietic stem cell transplantation. *Methods Mol Biol*. 2013;999:137–49.
39. Manasatienkij C, Chatchawin R. Clinical application of forensic DNA analysis: a literature review. *J Med Assoc Thai*. 2012; 95(10):1357–363.
40. Seattle Cancer Care Alliance. Chimerism-testing: chimerism testing/engraftment analysis. 2013 (retrieved from www.seattlecca.org/Chimerism-Testing.cfm; accessed September 16, 2013).
41. Jadus MR, Wepsic HT. The role of cytokines in graft-versus-host reactions and disease. *Bone Marrow Transplant*. 1992;10:1–14.
42. Krenger W, Ferrara JL. Dysregulation of cytokines during graft-versus-host disease. *J Hematother*. 1996;5:3–14.
43. Chao NJ, Schmidt GM, Niland JC et al. Cyclosporine, methotrexate, and prednisone compared with cyclosporine and prednisone for prophylaxis of acute graft-versus-host disease. *N Engl J Med*. 1993;329:1225–230.
44. Porter D, Antin J. Graft-versus-leukemia effect of allogeneic bone marrow transplantation and donor mononuclear cell infusions. In: Winter J, ed. *Blood Stem Cell Transplantation*. Norwell, MA: Kluwer Academic; 1996:57–85.
45. Mackinnon S, Papadopoulos EB, Carabasi MH et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood*. 1995;86:1261–268.
46. Bowden RA, Slichter SJ, Sayers MH et al. Use of leukocyte-depleted platelets and cytomegalovirus-seronegative red blood cells for prevention of primary cytomegalovirus infection after marrow transplant. *Blood*. 1991;78:246–50.
47. Leukocyte reduction for the prevention of transfusion-transmitted cytomegalovirus. Bethesda, MD: AABB; 1997. Association Bulletin #97-2.
48. Bowden RA, Slichter SJ, Sayers M et al. A comparison of filtered leukocyte-reduced and cytomegalovirus (CMV) seronegative blood products for the prevention of transfusion-associated CMV infection after marrow transplant. *Blood*. 1995;86:3598–603.
49. Preiksaitis JK. The cytomegalovirus-"safe" blood product: is leukoreduction equivalent to antibody screening? *Transfus Med Rev*. 2000;14:112–36.
50. Gratwohl A, Baldomero H, Aljurf M et al. Hematopoietic stem cell transplantation: a global perspective. *JAMA*. 2010;303:1617–624.
51. Gooley TA, Chien JW, Pergam SA et. al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med*. 2010;363:2091–2101.
52. Bhatia S, Robison LL, Francisco L et al. Late mortality in survivors of autologous hematopoietic-cell transplantation: report from the Bone Marrow Transplant Survivor Study. *Blood*. 2005;105:4215–222.
53. Chinen J, Puck JM. Perspectives of gene therapy for primary immunodeficiencies. *Curr Opin Allergy Clin Immunol*. 2004;4(6):523–27.



30 Morphologic Analysis of Body Fluids in the Hematology Laboratory

JERELYN WALTERS, BS
C. NANETTE CLARE, MD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. List the types of body fluids studied in the clinical laboratory, and describe the body cavities in which they are found.
2. List physical characteristics of various fluids.
3. Describe cell-counting and slide preparation techniques
4. List and describe the normal cells seen in each body fluid type.
5. Describe various artifact types that can be seen in body fluid preparations including those in cytocentrifuged specimens.
6. Describe the appearance of bacterial and fungal organisms in Wright-stained preparations.
7. List the differentiating morphologic features of benign and malignant cells on Wright-stained preparations.
8. List the types of crystals that can be found in joint fluids.
9. Describe routine hematologic methods for semen analysis.
10. Interpret automated lamellar body counts in amniotic fluid.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Identify the procedure used to obtain each type of body fluid.
2. Describe the production of serous, synovial, and cerebrospinal fluid (CSF).
3. Define and differentiate *exudates* and *transudates*, *chylous* and *pseudochylous* fluids.
4. Identify common cell types seen in CSF, serous, synovial, and broncoalveolar lavage fluids.
5. Evaluate the significance of microorganisms found in Wright-stained body fluid preparations and suggest methods to confirm them.
6. Compare and contrast the morphologic distinction between benign tissue cells and malignant cells in cytocentrifuged Wright-stained preparations of body fluids, and identify these cells when seen.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

7. Recognize erythrophagocytosis, and explain its significance in CSF.
8. Define *birefringence*, and explain its use in the identification of crystals in body fluids.
9. Identify and differentiate the various types of crystals that can be found in joint fluids, and associate them with particular disorders.
10. Given CSF parameters, differentiate a true hemorrhage from a traumatic tap.

Key Terms

Arachnoid mater
Ascite
Ascitic fluid
Birefringence
Bronchoalveolar lavage (BAL)
Central nervous system (CNS)
Cerebrospinal fluid (CSF)
Chylous
Dura mater
Effusion fluid
Exudate
Lamellar body
Meninges
Pericardial cavity
Pericardium
Peritoneal cavity
Peritoneum
Pia mater
Pleocytosis
Pleural cavity
Pseudochylous
Semen
Sperm
Synovium
Transudate

Background Basics

Although the study of body fluids is not generally included in textbooks of hematology, most clinical hematology laboratories have the responsibility to perform total cell counts and differential cell counts on body fluid samples. Although much of this chapter can be read independently of the other chapters, the chapter builds on the general knowledge of hematologic morphology, cell counting, and hematologic neoplasms. You should review the following concepts before beginning this chapter:

Level I

- Recognize the common types of normal cells found in the peripheral blood. (Chapters 1, 5, 7, 8)
- List the major types of neoplastic hematopoietic disorders. (Chapter 23)
- Describe and understand the use of the hemacytometer to perform manual cell counts. (Chapter 37)
- Describe the major types of lymphoma. (Chapter 28)

Level II

- Recognize reactive hematopoietic cells found in the peripheral blood. (Chapters 7, 8, 21, 22)
- Describe and recognize the hematopoietic precursors found in the normal bone marrow. (Chapters 4, 5, 7, 8, 38)
- Describe and identify the cells associated with the various types of acute (precursor) leukemia. (Chapters 26, 27)
- Compare and contrast the various types of lymphoproliferative disorders. (Chapter 28)

CASE STUDY

We will address this case study throughout the chapter.

Carolyn, a 51-year-old woman who is otherwise in her usual state of good health, has flulike symptoms. After two weeks, her cough and fever persist. Antibiotics were started, and 2 days later she has severe right-side chest pain and worsening shortness of breath. Radiologic studies show a large effusion in the right pleural cavity. A thoracentesis is performed, and 1 liter of thick, yellow fluid is aspirated.

As you read and study this chapter, think about the type of fluid this could be and the laboratory studies that should be done on the fluid to assist in diagnosis.

OVERVIEW

The hematology laboratory usually receives body fluids for cell counts and morphologic evaluation. The types of cells found and their concentration are helpful information for the clinician in arriving at a diagnosis. The cells that can be found in these fluids include white blood cells (WBCs) and red blood cells (RBCs) as well as tissue and tumor cells. The cytocentrifuge is used to prepare slide preparations for morphologic review, and Wright stain commonly is used to stain the cells on the slide. Identifying and differentiating the various types of cells seen in body fluids requires experience. However, all laboratory professionals who perform body fluid analysis should be able to differentiate the types of WBCs, normal tissue lining cells, and most malignant cells from benign cells. This chapter describes the sites where body fluids are found, the methods by which the fluids are obtained, and an explanation of why fluids

accumulate at these sites. The focus of the chapter is the hematologic analysis of these fluids and identification of the types of cells and inclusions that can be found in each fluid. The artifacts that can be found and the way to differentiate them from significant inclusions are also described and depicted. This chapter is presented in atlas format because this is the most helpful way to discuss the morphology of body fluid cells.

INTRODUCTION

The hematology laboratory plays an increasingly important role in the morphologic evaluation of body fluids. This is primarily because of the use of the cytocentrifuge, which markedly improves morphology over the previously used direct smear technique. The cytocentrifuge is an instrument used to prepare slides from body fluid specimens other than peripheral blood. The sample is centrifuged directly onto a glass slide and yields a concentrate of cells with excellent morphology. Properly prepared, a cytocentrifuge slide yields an approximate 20-fold concentration of cells.

The Wright-stained cytocentrifuge-prepared slide is made in the hematology laboratory to perform a differential of the nucleated cells present in the fluid. In addition, this slide is valuable in making many important diagnoses, both benign and malignant. Most malignant cells, including hematopoietic malignancies, carcinomas, and sarcomas, can be recognized microscopically. The hematopoietic malignancies are generally easier to diagnose from the Wright-stained slide than from the routine cytology preparation, which is prepared by alcohol fixation of cells and Papanicolaou staining. Another advantage of the slides made in the hematology laboratory is that they are prepared within an hour of specimen receipt, whereas cytology preparations take longer. Additionally, important nonmalignant findings, such as intracellular bacteria and fungi, which can be diagnosed on a Wright-stained slide, frequently are not seen on cytology slides. Hematologic slides, however, should not replace cytology preparations, which have better retention of nuclear detail and are superior to hematologic slides when attempting to determine the specific type of carcinoma or sarcoma present. Both techniques are necessary and aid in arriving at the most accurate diagnosis.

TYPES OF BODY FLUIDS

The body fluids discussed in this chapter are those commonly analyzed in the hematology laboratory, including pleural, peritoneal, and less commonly pericardial fluids that, as a group, are called *serous fluids*. They also include cerebral spinal fluid from the central nervous system (CNS) and synovial fluid from joint spaces. The medical techniques for obtaining these fluids (Table 30-1 ★) are, at times, inappropriately used to identify the type of fluid. For example, the laboratory can receive a fluid labeled thoracentesis fluid, but for accuracy of reporting results, the fluid should be identified as a pleural fluid. This chapter also reviews hematologic testing of less common fluids, including bronchial lavage fluid, semen for routine analysis, and amniotic fluid used to obtain lamellar body counts.

★ **TABLE 30-1** Sites of Origin of Body Fluids, Procedure to Obtain Fluid, and Type of Fluid

Anatomic Site	Procedure	Fluid Obtained
Pleural cavity	Thoracentesis	Pleural fluid
Pericardial cavity	Pericardial aspiration	Pericardial fluid
Peritoneal cavity	Paracentesis	Peritoneal fluid (ascitic fluid)
Joint space	Joint aspiration (arthrocentesis)	Synovial fluid
Spinal cord	Spinal tap, lumbar puncture	Cerebrospinal fluid

Serous Fluids

The pleural, pericardial, and peritoneal cavities are similar in structure and function. These cavities are often referred to as *spaces* (i.e., pleural space or peritoneal space) but are actually only potential spaces and do not normally contain any appreciable amount of fluid. These potential spaces are the minimal gap between the serous membrane structures that comprise the pleural, peritoneal, or pericardial membrane. The serous membranes are identified as the *parietal membrane*, which is the outer membrane, and the *visceral membrane*, which is the inner membrane closest to the internal organs. These membranes are composed of a continuous single layer of mesothelial cells and submesothelial connective tissue. Normally, there is just enough fluid between these two serous membrane surfaces to keep them moist. For example, the pleural space normally contains <15 mL of fluid. The fluid that forms in these spaces is an ultrafiltrate of plasma, which means chemical constituents such as protein and cholesterol can pass through the membranes, but cellular elements cannot. The parietal membrane produces the serous fluid that the visceral membrane absorbs at a dynamic rate. Fluid transfer across the pleural membranes is thought to be as high as 5,000 to 10,000 mL per 24 hours in normal adults. Fluid production depends on four factors known as *Starling forces*: capillary hydrostatic pressure, plasma oncotic pressure, lymphatic resorption, and capillary permeability.[1,2] Any pathologic state affecting one or several of these four factors can result in an abnormal collection of fluid. As a group, excess fluids that form in the pleural, peritoneal, or pericardial space are referred to as **effusion fluids**, although peritoneal effusion fluid is often referred to as **ascitic fluid** or **ascites**.

The **pleural cavities** (left and right) consist of the space between the lung and the inside portion of the chest wall. The pleural membrane's purpose is to provide a moist surface to minimize friction between the lung and chest wall as respiration occurs (Figure 30-1 ■).

The **peritoneal cavity** consists of the space between the inside of the abdominal wall and the outside of the stomach, small and large intestine, liver, and superior aspect of the urinary bladder and uterus. The kidneys are positioned posterior to the peritoneal membrane (the **peritoneum**) and are referred to as *retroperitoneal* (Figure 30-2 ■).

The **pericardial cavity** is the space between the outermost aspect of the heart and the innermost aspect of the pericardial sac. The **pericardium** is the membrane that provides a continuous covering of the pericardial cavity (Figure 30-1). With advances in imaging, pericardial fluids are seen infrequently in the laboratory.

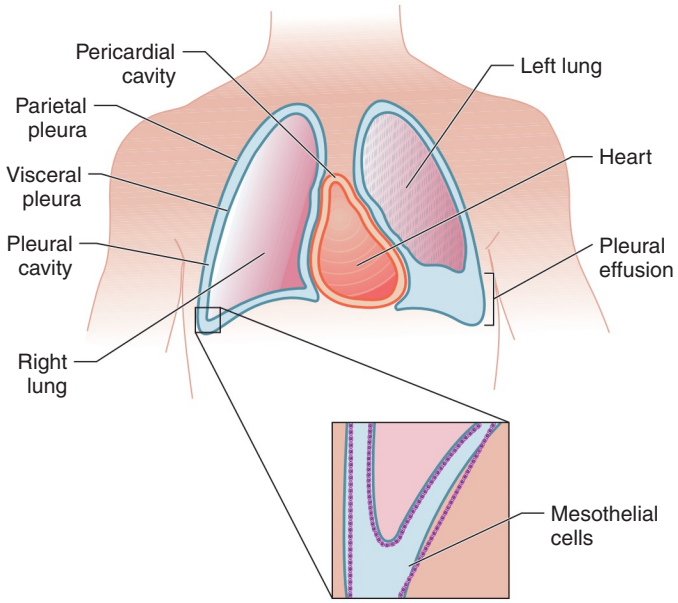


FIGURE 30-1 Diagram of right lung illustrating the pleural cavity and inset showing mesothelial cells of the parietal and visceral pleura. Illustration of heart shows the pericardial cavity. Diagram of the lung shows the abnormal accumulation of fluid (effusion) in the pleural cavity compressing the lower left lung.

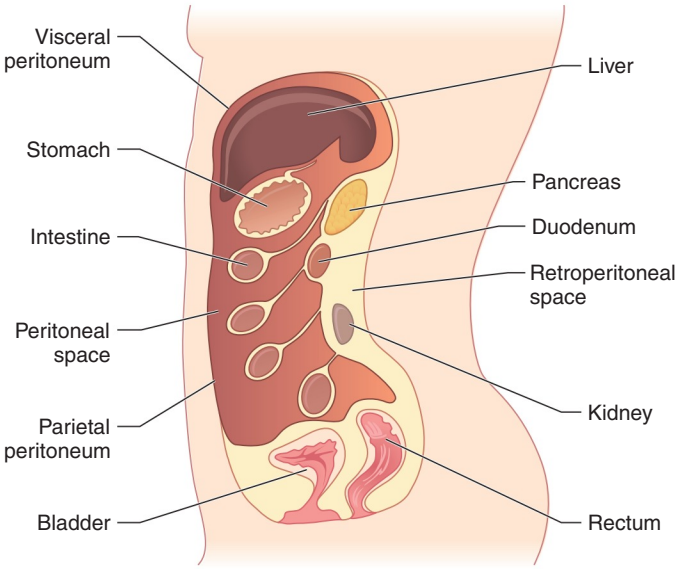


FIGURE 30-2 Abdominal cavity illustrating the peritoneal space between the inside of the abdominal wall and the outside of the liver, stomach, intestine, and dome of the bladder.

Effusion fluids are traditionally classified as transudates or exudates to aid in clinical diagnosis. An effusion can accumulate from a systemic disease state (**transudate**) or a primary pathologic state of the area (**exudate**). Transudates are frequently a result of increased capillary hydrostatic pressure as seen with congestive heart failure or of decreased plasma oncotic pressure as seen with hypoproteinemia because of nephrotic syndrome or liver failure. The capillary hydrostatic pressure and plasma oncotic pressure control the rate of filtration and reabsorption of the serous fluid. Changes in these pressures can cause fluid to accumulate, but the fluid is still an ultrafiltrate of plasma, resulting in an effusion fluid that is fairly clear and uncomplicated.

An exudate is formed by increased capillary permeability and/or decreased lymphatic resorption. Many different pathologic processes, such as bacterial infections, viral infections, neoplasms, and collagen vascular diseases, can cause an exudative effusion. These diseases can damage the serous membrane, rendering it incapable of providing an ultrafiltrate of plasma. As a result, plasma constituents normally filtered are allowed to seep into the fluid resulting in a more complicated fluid.

Although traditional criteria for differentiating transudates from exudates included specific gravity measurements and leukocyte counts, the current criteria for pleural effusions, known as *Light's criteria*, use serum and fluid measurements for protein and lactate dehydrogenase (LDH).[3] According to Light's criteria, if a pleural effusion meets one of the following criteria, it is classified as an exudate.

- Pleural fluid/serum protein ratio >0.5
- Pleural fluid/serum LDH ratio >0.6
- Pleural fluid LDH $>\frac{2}{3}$ the normal upper limit for serum

Current criteria for differentiating peritoneal effusions use a calculation known as the *serum–ascites albumin gradient (SAGG)*.[4] If the serum albumin minus the fluid albumin is equal to or greater than 1.1 g/dL the fluid is classified as a transudate. A result of <1.1 g/dL indicates an exudate.

Differentiation of transudate and exudate effusion fluids can assist the physician in determining whether the cause of the effusion is a systemic disease (transudate) or a primary pathologic local disease (exudate). Although hematologic results such as cell counts and cell types are not included in the transudate/exudate differential equation, they do provide significant information in discerning the cause of many exudates.

Cerebrospinal Fluid

The **central nervous system (CNS)**, consisting of the brain and spinal cord, is normally lined by special membranes referred to as *meningeal membranes*, or *meninges*, that protect it. The **meninges** consist of a relatively thick **dura mater**, the outermost membrane; a thinner **arachnoid mater**, the middle membrane; and an innermost **pia mater** that lies directly on the surface of the brain and spinal cord.

The **cerebrospinal fluid (CSF)** occupies the subarachnoid space between the arachnoid mater and pia mater and protects the brain and spinal cord from sudden changes in pressure, provides a stable chemical environment, and allows for the exchange of nutrients and waste products (Figure 30-3 ■). The choroid plexus cells and ependymal lining cells found in the ventricles produce the CSF. This CSF circulates through the ventricular system in the cerebrum, cerebellum, and brain stem and completely covers the surface of the brain and spinal cord. The CSF, a product of ultrafiltration and active secretion, is made at a rate of approximately 21 mL/hour[5] and is reabsorbed by the arachnoid cells. The total CSF volume in adults is 90–150 mL, but the constant production and reabsorption of nearly

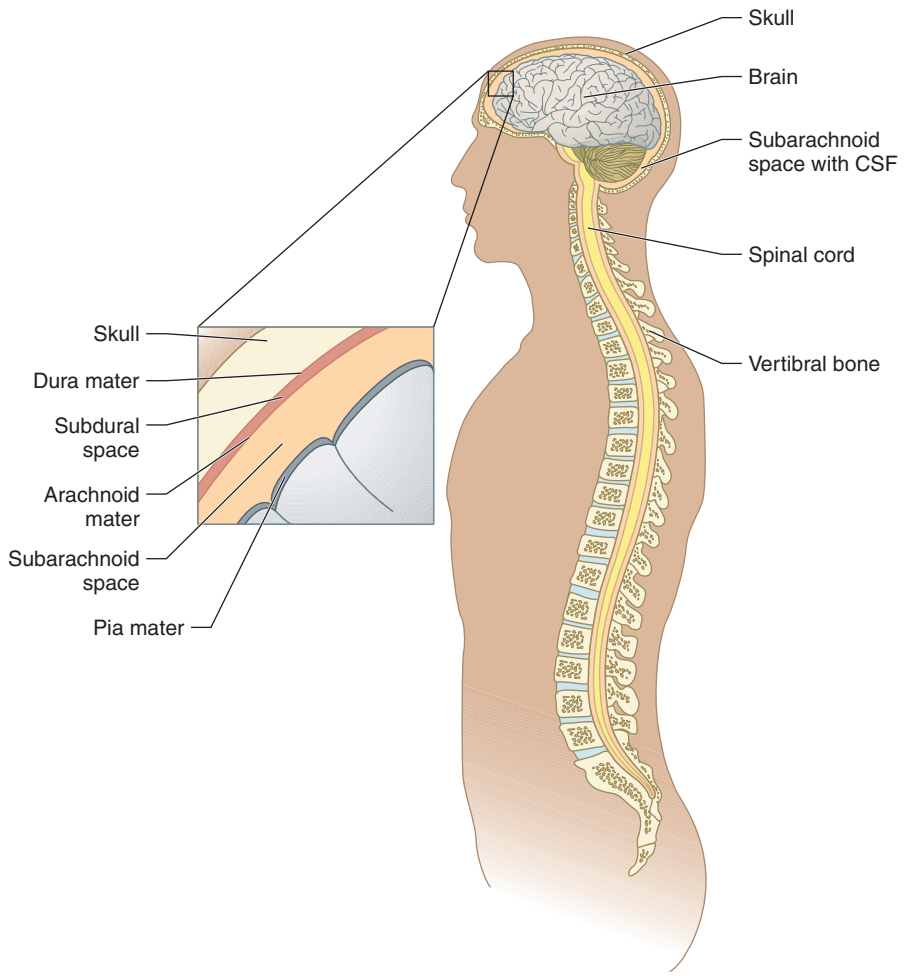


FIGURE 30-3 Central nervous system showing the subarachnoid space containing cerebral spinal fluid (CSF) covering the brain and spinal cord. Inset illustrates the meninges: dura mater, arachnoid mater, and pia mater.

500 mL per day results in a dynamic fluid that is replaced every 5 to 6 hours. Neonates have a CSF volume of 10–60 mL. The four primary reasons to test CSF in the laboratory are[2]:

- To detect meningitis
- To detect a subarachnoid hemorrhage
- To detect a malignancy
- To detect demyelinating disease

Meningitis is an inflammation of the meningeal membranes most often caused by viral, bacterial, or fungal infection in the CSF. Regardless of the cause, the universal finding in meningitis is an elevated leukocyte count. The presence of an elevated leukocyte count is called **pleocytosis**, a term unique to CSF. Determining the cause of meningitis is of great importance because bacterial meningitis can be life threating. Although serious, viral meningitis is rarely fatal. Fungal meningitis is more commonly seen in individuals with compromised immune systems.

Subarachnoid hemorrhage (SAH) is bleeding into the space between the two innermost meningeal membranes—the arachnoid membrane and the pia mater. SAH is a life-threatening event with a mortality rate as high as 25–50%. Thus, prompt diagnosis is critically important. The current gold standard for detection of an SAH is computed tomography (CT), which has a reported sensitivity and specificity approaching 100% if performed within 6 hours of the onset of symptoms (e.g., severe or "thunderclap" headache).[6] Unfortunately, the sensitivity and specificity decrease if testing extends beyond 6 hours. In cases when patients can delay seeking treatment or CT might not be readily available, the physician likely performs a lumbar puncture and submits CSF to the hematology laboratory for testing to assess the probability of an SAH. A patient with an SAH has significant RBCs in the spinal fluid. However, the diagnostic accuracy of detecting an SAH by examination of CSF is an imperfect science at best. A lumbar puncture can result in a "traumatic tap," meaning peripheral blood is introduced into the CSF sample during the puncture. It is estimated that 20% of lumbar punctures are traumatic, making it difficult to determine the actual source of the RBCs. See Table 30-2 ★ for traditional criteria for differentiating a traumatic tap from a true SAH. It should be noted, however, that the differentiating characteristics are not infallible because it is possible to have a traumatic tap overlying an SAH.[7,8]

★ **TABLE 30-2** Criteria for Differentiation of Subarachnoid Hemorrhage (SAH) and Traumatic Tap

Subarachnoid Hemorrhage	Traumatic Tap
Decreasing amount of blood in sequential tubes	Equal amount of blood in all tubes
Blood does not clot	Blood clots detected in 1 or more tubes
Xanthrochromia	Colorless supernatant
Negative D-dimer test[a]	Positive D-dimer test[a]

[a]Currently, no FDA-approved D-dimer test for CSF testing is used although a fair amount of published literature describing its use does exist.

Morgenstern LB, Luna-Gonzales H, Huber JC et al. Worst headache and subarachnoid hemorrhage: prospective, modern computed tomography and spinal fluid analysis. *Ann Emerg Med.* 1998;32:297–304; Julia-Sanchis ML. Rapid differential diagnosis between subarachnoid hemorrhage and traumatic lumbar puncture by D-dimer assay. *Clin Chem.* 2007;53:993

It must be noted that currently there is no D-dimer test that has been approved for CSF testing by the FDA, although a fair amount of published literature describing its use does exist.[6,9]

The detection of malignant cells in CSF can be critically important in both treatment and prognosis, including leukemic cells. Many current chemotherapeutic agents do not cross the blood brain barrier; thus, malignant cells harbored in the CNS can be a source of disease relapse. Intrathecal chemotherapy (injection directly into the spinal canal) is a therapeutic option in these cases. Malignant cells found in the CSF are often from metastatic disease, most commonly from breast and lung tumors. Primary brain tumors seldom involve the meninges, and the malignant cells from these tumors are not often seen in the CSF.

Examination of the CSF for the detection of demyelinating disease includes immunoglobulin and electrophoresis testing, which is typically not performed in the general hematology laboratory.

☑ CHECKPOINT 30-1

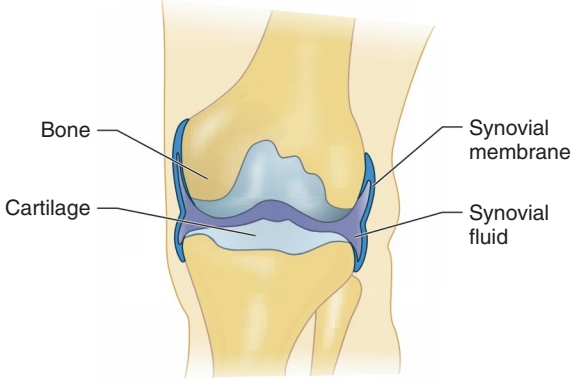
To obtain a sample of CSF for analysis, the needle must be inserted into what area of the central nervous system?

Synovial Fluid

Some bony joints of the body are lined with special membranes called the **synovium** that normally consists of a single layer of synovial cells (Figure 30-4 ■). The joint space contains synovial fluid that acts as a lubricant and a transport medium for nutrients to get to the joint's bone surfaces. Minimal fluid normally is present with the largest synovial joint, the knee, containing <4 mL. The synovial fluid is produced in part by the synovial cells and is an ultrafiltrate of plasma. Synovial fluid also contains a mucopolysaccharide called *hyaluronic acid*, which sometimes makes the fluid so thick that it hampers laboratory studies. A small amount of *hyaluronidase* powder can be added to an aliquot of synovial fluid to decrease the viscosity and allow accurate cell counting and proper slide preparation. Synovial fluids are not identified as transudates or exudates for diagnostic purposes but are classified into groups based on laboratory findings (Table 30-3 ★). The two conditions in which laboratory results are essentially diagnostic of specific joint disease are a positive gram stain and culture indicating infection, and the identification of monosodium urate or calcium pyrophosphate dehydrate crystals indicating a crystal-induced arthritis.[1]

Bronchoalveolar Lavage (BAL)

A **bronchoalveolar lavage (BAL)** is not a normally occurring fluid but is obtained by a diagnostic procedure of infusing and removing a sterile saline solution into the alveolar and bronchial airspaces of the lung via a bronchoscope. The lavage procedure allows harvesting the cellular and noncellular elements of the lower respiratory tract; it is a valuable tool in diagnosing interstitial lung diseases, infections, and malignancies. BAL is especially helpful in diagnosing infections caused by difficult-to-culture organisms such as *Pnemocystis jirovecii* (formerly *P. carinii*). Other conditions that have fairly specific



■ **FIGURE 30-4** Knee joint illustrating synovial membrane lining and synovial fluid.

★ **TABLE 30-3** Diagnostic Joint Fluid Groups

Group I Noninflammatory	Group II Inflammatory	Group III Septic	Group IV Crystal Induced	Group V Hemorrhagic
Osteoarthritis, traumatic arthritis, other degenerative joint disorders	Rheumatoid arthritis, lupus erythematosus, other immunologic disorders	Infection: bacterial, mycobacterial, or fungal	Gout, pseudo gout	Traumatic injury, hemophilia

findings in BAL include alveolar proteinosis, pulmonary hemorrhage syndrome, eosinophilic pneumonia, hypersensitivity pneumonitis, and sarcoidosis.[10]

The hematologic analysis of BAL centers on the enumeration and morphologic differentiation of the nucleated cells present. The concentration of cells varies with the quality of the sample (amount of saline infused and retrieved) but as with other body fluids, the type of cell present typically has more importance than the cell count itself. The majority of cells normally present are macrophages (>80%) and a few hematopoietic cells, primarily lymphocytes (considered a contaminant caused by the procedure).[11] Both fungal and bacterial microorganisms can be seen in the hematology-stained smear and should be noted, but proper identification of these elements should be left to the microbiology laboratory.

HEMATOLOGIC ANALYSIS OF BODY FLUIDS

Specimen Collection and Handling

Normal serous synovial fluid does not clot. However, body fluids are submitted to the laboratory because of a suspected abnormal condition. That, along with the possibility of peripheral blood contamination introduced in the process of obtaining the sample, increases the possibility of clotting occurring in the sample. To ensure accurate cell counts, the preferred sample for hematology analysis is one submitted in an anticoagulant tube containing EDTA or sodium heparin. There are anecdotal reports of lithium heparin forming rhomboid crystals that can be confused with calcium pyrophosphate dehydrate crystals, so it should be avoided as an anticoagulant for synovial fluids.[12]

Viscous synovial fluids should be treated with hyaluronidase before performing cell counts or preparing cytocentrifuge slides. The volume of fluid received by the laboratory should be recorded.

Normal CSF also does not clot, but it is never submitted in an anticoagulant tube because the presence of a clot is an indicator of a traumatic tap rather than a subarachnoid hemorrhage.[1] CSF is typically collected in a sequential series of three or four nonanticoagulant sterile tubes. The last tube collected should be used for hematologic analysis because it is the least likely to be affected by any peripheral blood contamination as a result of performing the lumbar puncture. Knowing the sequence in which the tubes were collected to differentiate a subarachnoid bleed from a traumatic tap is essential. The total volume of CSF and number of tubes received in the laboratory should be recorded.

BAL samples do not require anticoagulants, and a portion of the lavage is usually submitted to the hematology laboratory in a sterile container. Whereas there is no published consensus at this time for the laboratory handling or processing of BAL, it is generally agreed the sample should not be filtered to remove any interfering mucus because this will result in loss of cells.[1] Samples can be treated with a commercially available mucolytic agent such as Mucolexx™ to digest the mucus and allow accurate cell counting and cytocentrifuge slide preparation.[13]

Clotted samples of any type of body fluid negatively affect the accuracy of both cell count and differential results. However, because body fluids are considered "irretrievable" samples, clotted samples should not be summarily rejected for analysis. Small fibrin clots can be removed from the sample and the cell count reported as "approximate, small clots present." The approximate count can be helpful if it indicates an increased cell count and, in truth, the type of cell present often has more importance than the actual cell count. Cell counts should not be performed on samples that are solidly clotted, but a valid attempt should be made to get some cells on a slide either by cytocentrifuging any free liquid or by a touch-prep technique.

Physical Characteristics

The clarity and color of all fluids should be noted and reported. Clarity can be graded simply as clear, hazy, or cloudy. Some laboratory professionals use the ability to read print through a sample to differentiate hazy from cloudy. Color can vary widely from colorless to yellow, pink, red, or brown. Samples can also be described as grossly bloody.

CSF is normally clear and colorless. As few as 200 WBCs/mcL (μL) or 400 RBCs/mcL can cause a hazy appearance. CSF that is bloody requires examination of all tubes received to aid in differentiating a traumatic tap from a subarachnoid hemorrhage. The presence of blood because of a traumatic tap decreases as the fluid is collected in sequential tubes; thus, the last tube collected will be less bloody than the first tube collected. An equal amount of blood in all tubes is suggestive of a subarachnoid hemorrhage. Some laboratories perform RBC counts on the first and last tube to aid in identifying the cause of the bloody CSF. Having the total RBC count in the first tube collected is significantly higher than that in the last tube collected suggests a traumatic tap.

CSF should always be examined for xanthochromia, a yellow, orange, or pink coloration of the CSF, often caused by the presence of breakdown products of red blood cells; it is usually thought to indicate true CNS hemorrhage (Figure 30-5 ■). If the CSF is cloudy or bloody, an aliquot of fluid should be centrifuged and the supernatant examined for xanthochromia. Xanthochromia appears within in several hours of the bleed and can persist for a week or more. Xanthochromia also can occur if a grossly bloody fluid from a traumatic tap sits for some time before it is centrifuged or the amount of plasma accompanying the blood introduced during collection is sufficient to color

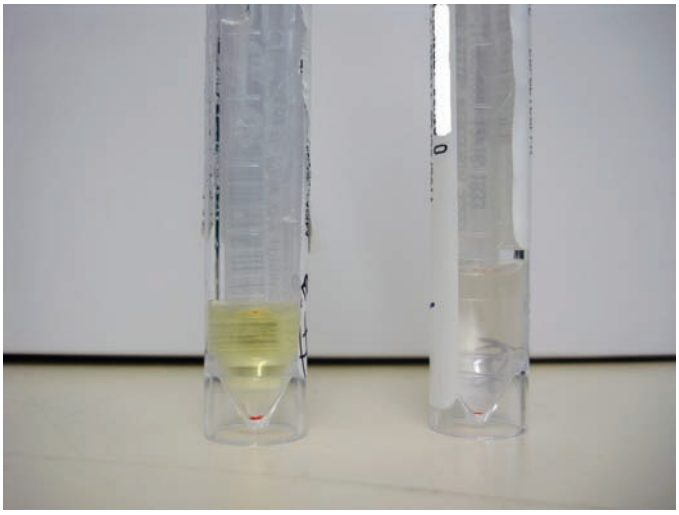


FIGURE 30-5 CSF samples showing xanthochromic (left) and colorless (right) supernatant.

the supernatant. Additionally, patients with very high serum bilirubin levels can have spillover into the CSF, causing the fluid to appear xanthochromic. More than 90% of patients with a serum bilirubin of 10 to 15 mg/dL have xanthochromic CSF. Because of infection or inflammatory conditions, high CSF protein levels (>150 mg/dL) can also exhibit xanthochromia.[1] Spectrophotometric methods for a more accurate detection of xanthochromia have been proposed, but recent literature supports visual examination as a more clinically appropriate method.[14] Long considered one of the better indicators of a subarachnoid hemorrhage, xanthochromia does have limitations in its predictive accuracy and can indicate a previous rather than current bleed.

Normal serous fluids (pleural, peritoneal, pericardial) are clear and pale yellow. Increased cellular elements, infection, or malignancy can cause hazy or cloudy fluids. Bloody fluids can be caused by a traumatic tap, pulmonary or intestinal infarction, malignancy, or trauma. It is generally not necessary to examine the supernatant of serous fluids unless a chylous fluid is suspected.

A **chylous** effusion has a characteristic milky, opaque appearance that remains in the supernatant after centrifugation. Chylous effusions result from lymphatic vessel leakage and are not commonly seen. In the pleural cavity (chylothorax), this results from leakage of the major thoracic duct. In the peritoneal cavity, chylous effusions result from blockage of the lymphatic vessels. In both the pleural and peritoneal cavities, chylous effusions most often result from a malignancy such as lymphoma or carcinoma or from trauma. This type of fluid is rich in chylomicrons, has elevated triglycerides, normal cholesterol, and its predominant cells are lymphocytes.[2,15] A **pseudochylous** effusion is also milky and results from a chronic, long-standing effusion because of conditions such as tuberculosis and rheumatoid pleuritis.[2] Pseudochylous effusions do not contain chylomicrons and have elevated cholesterol and low triglycerides. Cholesterol crystals are commonly seen, as is a mixed reactive cell population with many inflammatory and necrotic cells.

Synovial fluid is normally pale yellow, clear, and viscous. Hazy or cloudy synovial fluid can be the result of infection, inflammation, or crystal formation. Bloody synovial fluid is often the result of trauma or traumatic tap but can be seen in hemophilic hemarthrosis and poorly controlled anticoagulation therapy.

Viscosity is an important component of synovial fluid. The high viscosity results from the glycoproteins hyaluronic acid and lubricin produced by the synovial lining cells, and these glycoproteins lubricate the joint. As seen in rheumatoid arthritis and other inflammatory conditions, inflammation can cause a decrease in the viscosity often to the point that the synovial fluid has the consistency of a serous fluid. Viscosity can be assessed at the bedside or in the laboratory by performing the simple string test. Synovial fluid with normal viscosity will form a string >5–10 cm in length or longer when dripped from a syringe or disposable transfer pipet. Inflammatory synovial fluid forms small drops like water.[16] Viscosity is typically reported as normal or decreased.

The color and clarity of BAL samples can have limited clinical correlation because of variances in volume retrieved and concentration, but should be reported none the less.

CASE STUDY *(continued from page 573)*

Radiologic studies show a large effusion in the right pleural cavity. A thoracentesis is performed, and 1 liter of thick, yellow fluid is aspirated. Laboratory studies show a total protein of 4.5 g/dL (serum = 6 g/dL), lactate dehydrogenase 40 U/L (serum = 50 U/L), and total leukocyte count of 20,000/mcL with 90% segmented neutrophils, 10% histiocytes, and many degenerating cells.

1. Is this a transudate or exudate?
2. Is this a chylous fluid?

Cell Counting

Cell counts of body fluids should be performed as soon as possible because of the risk of WBC lysis. WBCs, especially neutrophils, are less stable in body fluids because they are not in the usual nutritive plasma environment where they normally exist.

A common rule is to perform the cell count within 1 hour of collection. This 1 hour limit is critical for accurate CSF analysis because studies have shown significant WBC loss, particularly neutrophils, at 1 hour postcollection.[17] Serous and synovial fluids have been shown to have improved cellular stability when refrigerated for 24 hours, but these guidelines may not apply to fluids that form because of sepsis or inflammation.

Cell counts can be performed by manual or automated methods. Automated methods provide more efficiency and precision compared with manual methods. Regardless of the method used, the WBC count performed on body fluids is more correctly reported as a total nucleated cell count (TNCC). Monocytes, macrophages, normal tissue lining cells, and some malignant cells often have the same cellular size and nuclear content, making it impossible to count just the WBCs; thus, all nucleated cells should be included in the count. Cell counts performed on CSF traditionally include both the TNCC and the RBC count because the RBCs can be helpful in detecting a subarachnoid

hemorrhage. The RBC count can be performed on both the first and last tube collected when there is suspicion of a traumatic tap as an aid in differentiating the two conditions. RBC counts are of limited use in serous and synovial fluids and are not performed routinely.[2] When necessary, RBC counts in grossly bloody or hemorrhagic fluids should be performed by automated methods. A hematocrit can be more helpful in these cases.

Synovial fluid should be treated with hyaluronidase before cell counting or slide preparation. A small amount of dry hyaluronidase powder pinched between two clean applicator sticks should be added to a 0.5–1 mL aliquot of the fluid.[18] The sample is then mixed gently for 20–30 seconds until it liquefies. If the sample aliquot is still viscous, an additional small amount of hyaluronidae powder can be added. The dry powder will not cause a dilutional effect. Cell counting should be performed immediately following hyaluronidase treatment.

Mucoid BAL samples should be treated with a mucolytic agent before performing the cell count and preparing the slide. Mucolytic agents are usually liquid and can add a significant dilution to the fluid. This dilution must be taken into consideration when calculating the final cell count result.

Manual Cell Counts

Manual cell counts are performed using a hemacytometer. Disposable hematometers, such as the C-chip™ (Web Figure 30-1), are preferred over the standard glass Neubauer chamber (Web Figure 30-2). The disposable hemacytometer has an integral coverslip, is for single use, and avoids the hazards of cleaning glass hemacytometers. The Neubauer hemacytometer consists of a glass counting chamber and a separate glass coverslip that must be properly placed for accurate counting. The hemacytometer has two counting chambers, one on each side. Each counting chamber is divided into nine large squares that are further subdivided into smaller squares. Each of the nine large squares measures 1 mm^2. The coverslip gives the counting chamber a depth of 0.1 mm; thus, the total volume of each counting chamber is 0.9 mm^3 (Chapter 37 describes detailed use of a hemacytometer).

Clear fluids can be counted undiluted. The pipet used to insert the fluid into the counting chamber can be rinsed with a stain such as new methylene blue before drawing up the fluid to assist in differentiating RBCs from WBCs when performing the cell count. To prevent any dilutional effect, the stain should be thoroughly expelled from the pipet before drawing the sample. Sufficient stain clings to the wall of the pipet to stain the WBCs, allowing more accurate identification. Refer to Web Figure 30-3 for an unstained mixture of RBCs and WBCs on a hemacytometer and to Web Figure 30-4 for the same fluid aspirated with a pipet rinsed with new methylene blue and plated on the hemacytometer.

Dilutions should be made for hazy, cloudy, or bloody fluids based on an estimate of cellularity that can be performed by observing a cover-slipped drop of fluid on a slide. Dilutions can be made with sterile saline or isotonic instrument diluent for counting both RBCs and WBCs. Samples with increased RBCs can make it difficult to identify the WBCs. These samples can be diluted with a commercially available diluent containing acetic acid (to lyse the RBCs) or a combined acetic acid/dye solution (to stain the WBCs) such as Turk's solution. Dilutions must be made with accurate pipetting devices (Table 30-4 ★).

★ **TABLE 30-4** Example of Suggested Dilutions for Manual Cell Counts in Hazy, Cloudy, and Bloody Body Fluids

Hazy (1:2)	Cloudy (1:5)[a]	Bloody (1:10)
1 part fluid	1 part fluid	1 part fluid
1 part diluent	4 parts diluent	9 parts diluent

[a]For example, for a 1:5 dilution, use 100 mcL of fluid and 400 mcL of diluent.

Both chambers (sides) of the hemacytometer should be plated with the fluid so that the count is performed in duplicate. If a dilution is necessary, duplicate dilutions should be used to fill each side of the chamber to reduce potential error. The cell counts on each side should match within 10 cells on cell counts <100/mcL and 10% on cell counts >100/mcL.[19]

The average number of cells counted on both sides is used to calculate the final result. The area of the hemacytometer counted is determined by the cellularity of the sample. Low cell counts require counting all nine squares on each side of the hemacytometer. Higher cell counts can be identified by counting the four large corner squares (WBC area on each side). If numerous RBCs are present, as in a bloody CSF, the usual RBC area (five small squares within the center large square) can be used to count the red blood cells (1/5, or 0.2 of 1 mm^2). The laboratory should provide guidelines indicating acceptable dilutions and the area to be counted based on cellularity (Web Table 30-1).

The cell count is calculated using the standard hemacytometer formula by taking into account the dilution made, if any, and the area counted. The formula for calculating hemacytometer cell counts is

$$\text{Total cell count (cells/mm}^3) = \frac{\text{Mean number of cells on both sides} \times \text{Reciprocal of dilution}}{\text{Number of large squares on one side} \times \text{Area of square} \times \text{Depth}}$$

EXAMPLE

The number of cells counted in four large corner squares on each side of the hemacytometer:

Side 1: 115
Side 2: 125
Mean of cells counted: 120
Dilution = 1:5
Area counted: four large squares (4 mm^2)
Depth: 0.1 mm

$$\frac{120 \times 5}{4 \times 1\ mm^2 \times 0.1\ mm} = 1{,}500\ cells/mm^3\ (1.5 \times 10^9/L)$$

A second person should verify manual calculations performed in the laboratory whenever possible.

Quality control (QC) should be performed for manual body fluid cell counts. Acceptable QC methods include commercially available controls or a procedural control. An acceptable procedural control is to correlate the nucleated cell count with the number of nucleated cells seen per field on the properly made cytocentrifuge-prepared slide.[20] As a general rule, a properly prepared cytocentrifuge slide concentrates the cells 20-fold. Thus, a fluid sample with a TNCC of 3/mcL should have <100 nucleated cells on the cytocentrifuge slide. If a 100-cell differential can easily be performed on the slide, the TNCC result of 3 cells/mcL should be suspect. Conversely, a TNCC of 30/mcL should provide sufficient cells on the cytocentrifuge slide to easily perform a 100-cell manual differential.

Automated Cell Counts

Automated body fluid cell counts can be performed on a variety of current generation hematology analyzers[21,22] (Chapter 39). It is imperative to know and understand the acceptable fluid types, analytic measurement range, clinical reportable range, and result interpretation for the analyzer in use. As with manual cell counts, synovial fluid should be treated with hyaluronidase before automated cell counting, and BAL samples should be treated with a mucolytic agent.

Restrictive low linearity limits on some analyzers can preclude analysis of clear, colorless fluids such as CSF, but more cellular fluids can usually be analyzed. For example, if the lower limit of an instrument's analytic measurement range for nucleated cells is 100/mcL, the clinical reportable range for serous and synovial fluids could be <100/mcL because <100 nucleated cells is well within the normal range for these fluids. Newer hematology analyzers have dedicated body fluid channels that use fluorescent dyes and extended count times to achieve an analytical measurement range accurate to one nucleated cell per microliter.[23] Bloody fluids can be analyzed on most hematology analyzers. Regardless of the cause of the bloody sample—traumatic tap or hemorrhagic condition—the blood present is peripheral blood, which these instruments were designed to analyze.

Interpreting acceptability of results is critically important. Body fluids often contain debris and noncellular elements that can interfere with accuracy. The scattergram or histogram accompanying the cell count must be examined for any interference.

See Web Figure 30-5, for examples of acceptable and unacceptable body fluid cell counts performed on an analyzer with a dedicated body fluid channel. Because scattergrams and histograms associated with cell counts vary by analyzer, it is essential to understand and appropriately interpret results generated by the particular analyzer in use.

Cell Count Reference Intervals and Correlation with Disease

The number and types of cells found in body fluids is important information that is used with clinical findings to help make a diagnosis.

Cerebrospinal Fluid (CSF)

The reference interval for WBCs in CSF is 0–5 cells/mcL for adults and 0–30 cells/mcL for neonates.[2,7] The higher upper reference interval seen in neonates decreases with age, so the reference interval for children up to 1 year old is 0–20 cells/mcL and 0–10 cells/mcL from age 1 year to adolescence.[3] In both adults and neonates, the predominant WBCs normally present are monocytes and lymphocytes.

The most important considerations for the interpretation of results are the types of WBCs present and the correlation with clinical findings. An increased WBC count in CSF indicates meningitis. Increased counts with predominantly neutrophils are associated with bacterial meningitis, whereas those with predominantly lymphocytes are associated with viral meningitis. Normally, RBCs are not present. The presence of RBCs indicates a subarachnoid hemorrhage or blood introduced by a traumatic tap.

The total WBC count cannot be interpreted without the total RBCs count. When a specimen is obtained as a traumatic tap, the WBCs and RBCs reflect the same WBC/RBC ratio as the patient's peripheral blood. A general rule is to expect 1 to 2 WBCs for every 1000 RBCs in the CSF in this situation. For example, if the total WBC count in a CSF specimen is 10/mcL and the RBC count is 10,000/mcL, no significant increase of WBCs (pleocytosis) is evident. If, however, the total RBC count is 100/mcL with a WBC count of 10/mcL, a significant increase of WBCs indicates a pathologic state.

Serous Fluids: Pleural, Peritoneal, Pericardial

Normal serous fluids have a total nucleated cell count <1000/mcL. The cells are predominantly mononuclear cells and can include a few mesothelial lining cells. Increased cell counts with a predominance of neutrophils suggest infection. An increased cell count with a predominance of lymphocytes is associated with a lymphoproliferative disorder, tuberculosis, or a chylous effusion. RBCs are not normally seen in serous fluids. Bloody serous fluid can result from a malignancy, trauma, or a traumatic tap.

Synovial Fluids

Normal synovial fluid has a total nucleated cell count of <200/mcL with predominantly mononuclear cells. Increased cell counts are associated with inflammation and infection. Certain diagnoses can be suspected when comparing the total WBC count with percent of segmented neutrophils present; however, there is significant overlap, and morphologic examination by cytocentrifuge preparations can be extremely helpful[24,25] (Web Table 30-2).

Joint fluids that have a total WBC count of 50,000 to 200,000/mcL suggest an infectious or crystal-induced etiology. If the differential count shows 90% or more segmented neutrophils, an infectious agent is most likely, and cultures must be obtained. Microorganisms can be seen in joint fluid if present in sufficient numbers and have the same morphology as previously described. Bacterial organisms are more common, and pathogenic yeasts are seen only rarely.

When the total WBC counts is in the range of 2,000 to 200,000/mcL with >50% neutrophils in the differential count, entities such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Reiter's syndrome should be considered.

Bronchoalveolar Lavage

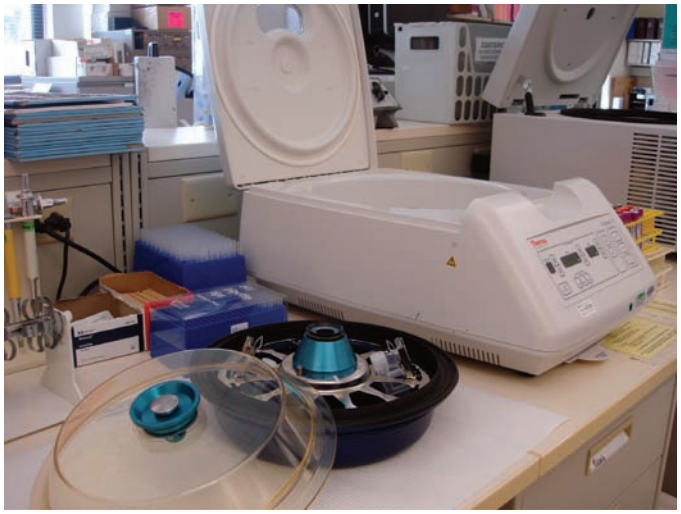
Reference intervals for nucleated cell counts in BAL samples are difficult to determine because the concentration depends on the volume of fluid infused and the return yield. Studies performed on normal subjects show a range of nucleated cell counts of 100–400/mcL.[26] Microscopic findings in BAL samples have more importance than the cell count itself.

Nucleated Cell Differential

Body fluid analysis requires not only enumeration of cells but also microscopic examination. The types of cells present as well as artifacts and crystals can be identified microscopically. In many cases, the types of cells present can be more important than the cell count. Morphologic examination and differentiation of cells require concentration techniques that preserve cellular integrity and morphology.

Cytocentrifuge Slide Preparation

Cytocentrifugation is a method that concentrates cells in a monolayer on a defined area of a slide with minimal morphologic distortion. A cytocentrifuge is a centrifuge with a bowl or rotor, usually removable, that can hold multiple slide assemblies (Figure 30-6 ■). The cytocentrifuge slide assembly consists of a funnel-shaped chamber, an absorbent filter material, and a slide. A clip holds the components together. Disposable chambers with attached filter material are available as are commercially available slides with pre-marked zones to identify the area where the cells are deposited. Slides should be made in duplicate, or commercially available dual chambers and slides should be used (Figure 30-7 ■). As with cell counts, slides should be made as soon as possible to avoid cellular degeneration and viscous synovial samples should be treated with hyaluronidase before slide preparation. To produce the best slides for morphologic examination, the amount of fluid used should be based on the cell count. Specific manufacturer instructions should be followed, but a general guideline is given in Web Table 30-3. Fluid should be added to the chamber with a disposable pipet. A drop of sterile 10–22% albumin should be added to the chamber when preparing slides on serous fluids and CSF. The albumin helps preserve cellular morphology in low protein samples and improves the adhesion of cells to the slide. Albumin should not be added to synovial samples because they have a high protein content. To obtain suitable slides for bloody samples, dilution of the RBCs to <5000/mcL is recommended.[1] In samples with low nucleated cell counts, this can be counterproductive because doing so can result in too few nucleated cells available. An alternate option for bloody serous fluid and CSF is to make a small dilution using 10% acetic acid to lyse the RBCs. Acetic acid should never be added to synovial fluids because it can cause precipitation of the proteins.



■ **FIGURE 30-6** Cytocentrifuge with removable head.

■ **FIGURE 30-7** Cytocentrifuge slide assembly with disposable dual chamber cytofunnel.

Cytocentrifuges with adjustable speed, time, and acceleration can be adjusted for optimum slide preparation on various fluids. As a general rule, serous fluids and CSF samples should be cytocentrifuged at a low speed (600 rpm) for 10 minutes with low acceleration. Synovial fluids can be cytocentrifuged at the same speed and time settings, but high acceleration, if available, can be helpful in harvesting cells in these samples.

Care must be taken when disassembling the slide assembly following cytocentrifugation. After removing the clip, the sample chamber must be pulled directly away from the slide. Allowing the chamber to slide up or down the slide will disrupt and remove the cells. Allow the slide to dry before staining with a Wright or Wright-Giemsa stain.

Manual Slide Preparation

Cytocentrifugation is the gold standard for body fluid slide preparation. However, there may be rare instances when a cytocentrifuge is not available or functional, in which case a smear must be made manually. Samples with low nucleated cell counts (<1,000 or 2,000/mcL) should be concentrated before making the smear. An aliquot (1–2 mL) of the fluid can be centrifuged to concentrate the cells in a button on the bottom of the tube. Centrifuging at a fairly low speed (1000–1500 rpm) for 10 minutes causes less disruption of the cells than centrifuging at a higher speed. The supernatant is removed and the button of cells is gently resuspended. One drop of 10–22% albumin should be added to the resuspended cells for serous and CSF samples. A drop of the concentrated cells can then be used to prepare a wedge smear similar to a peripheral blood smear; the wedge smear can be made directly from the sample on highly cellular fluids.

Cell Types and Microscopic Findings Common to All Fluids

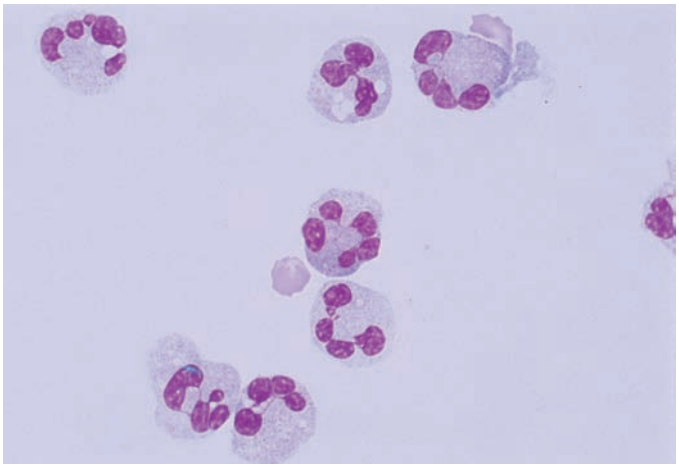
Segmented neutrophils (segs) are frequently seen in the pleural, pericardial, and peritoneal fluids in varying numbers. The neutrophils have the same appearance as in peripheral blood smears. However,

cytocentrifuge artifactual changes sometimes can be seen with nuclear segments being thrown to the periphery of the cytoplasm, creating a "windmill" and hypersegmented appearance (Figure 30-8 ■; all slides in this chapter were cytocentrifuge-prepared body fluids, Wright stained, magnification 1000×, unless otherwise noted). Degeneration of neutrophils is seen more frequently in body fluid samples than in peripheral blood smears. Peripheral blood cells, especially neutrophils, are less stable in body fluids because they are not in their normal plasma environment. Studies have shown that in CSF, up to 32% of the neutrophils can be lost in 1 hour because of degeneration.[17] The dying cells show cytoplasmic vacuolization and separation of nuclear segments with dense-staining chromatin (Figure 30-9 ■). These cells can be mistaken for nucleated RBCs and even yeast organisms. Neutrophilic precursors, such as promyelocytes, myelocytes, and metamyelocytes, are not commonly seen, but if present they can represent a chronic inflammatory process or true marrow disorder, such as myeloproliferative disorders, myelodysplastic states, and leukemia. Myeloblasts are usually seen only in the latter three.

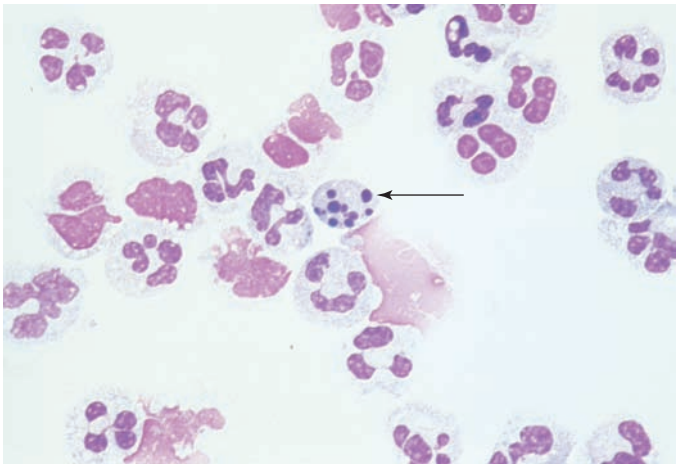
Lymphocytes are frequently present in all types of fluids in variable numbers. The lymphocytes vary in morphology from small to large and transformed (reactive). In cytocentrifuge preparations, the lymphocyte nucleoli can be artifactually more prominent than in peripheral blood smears, the nuclear shape can be irregular, and the cytoplasm can have artifactual projections[3] (Figures 30-10 ■ and 30-11 ■). If neoplastic lymphocytes (leukemias, lymphomas) are present, the morphology depends on the type of neoplasm, and the cells are usually homogeneous in appearance. Flow cytometry or immunoperoxidase techniques can be helpful in distinguishing benign versus malignant lymphocytes.

Monocytes in body fluids can appear similar to that seen in peripheral blood smears or can be larger with abundant, vacuolated cytoplasm (histiocyte), or they can have actual phagocytosed material (macrophage, phagocyte) (Figures 30-12 ■ through 30-14 ■). The distinction among the three morphologic types (monocytes, histiocytes, and macrophages) is not clinically important, and these cells can be reported as a combined population of mono/macrophages. In some cases, however, the phagocytosed material or organisms can be diagnostically important. Vacuoles that develop in phagocytic cells can fuse into a single large vacuole resulting in a "signet ring" appearance with the nucleus flattened against the cell membrane (Figure 30-15 ■). These signet ring cells can be seen in both benign and malignant conditions. Only a few monocytes are present in CSF, and histiocytes/macrophages usually are seen in the CSF only in pathologic states. Plasma cells are not seen in normal fluids and usually are present only in chronic inflammatory disorders (Figure 30-16 ■).

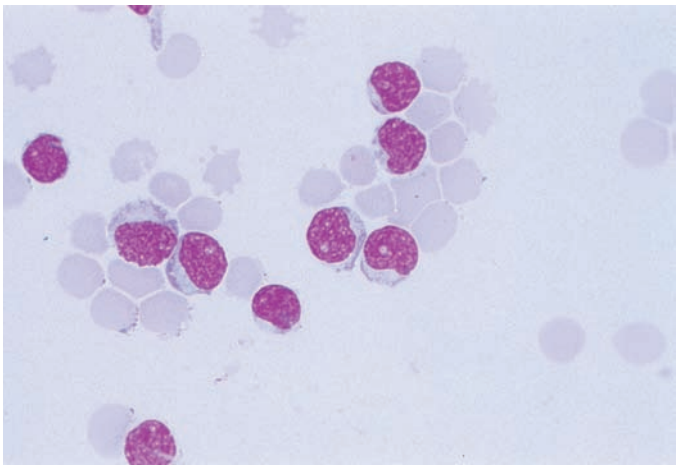
Eosinophils, basophils, and mast cells can be present in small numbers in pleural, pericardial, peritoneal, or joint fluids. Increased numbers of these cell types are seen in various disorders and can correlate with peripheral blood eosinophilia or basophilia.[4] Eosinophils are frequently seen in nonspecific or idiopathic effusions and can be present in effusions caused by various infectious agents, malignancies, and connective tissue disorders. Mast cells can be distinguished from basophils because mast cells have a round (not segmented) nucleus and a higher number of cytoplasmic granules than basophils. The granules in mast cells are smaller than those seen in basophils (Figures 30-17 ■ and 30-18 ■). An increase in the



■ **FIGURE 30-8** Artifactual change in neutrophils showing nuclear lobes thrown to the periphery of the cytoplasm. (Slides in this chapter are all cytocentrifuge-prepared body fluids, Wright stained, magnification 1000× unless otherwise noted).



■ **FIGURE 30-9** In the center (arrow) is a degenerating neutrophil with separation of nuclear lobes and dense staining of the chromatin.



■ **FIGURE 30-10** Artifactual change in lymphocytes with overly prominent nucleoli.

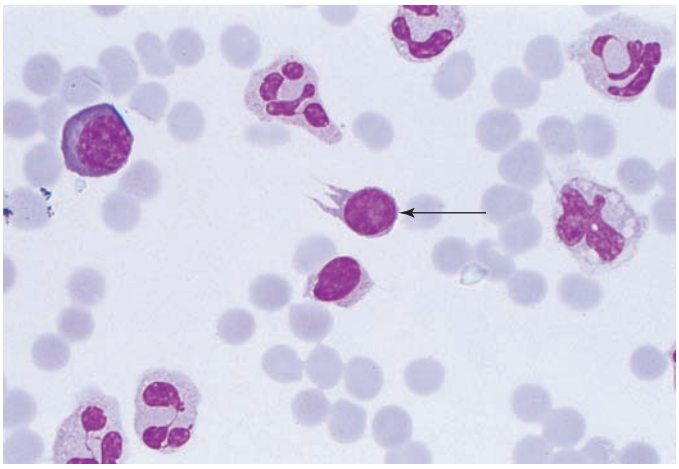


FIGURE 30-11 Artifactual change in lymphocytes with cytoplasmic projections.

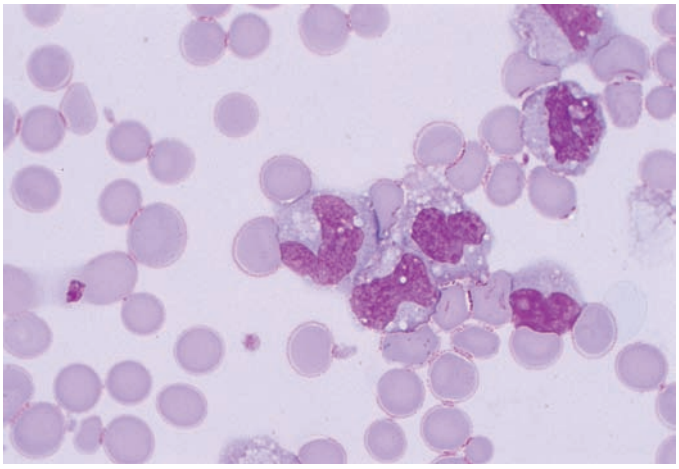


FIGURE 30-12 Monocytes in pleural fluid.

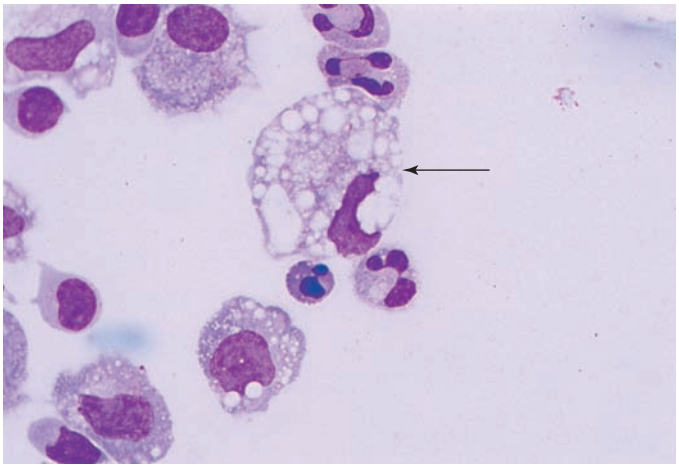


FIGURE 30-13 Histiocyte (arrow) in pleural fluid.

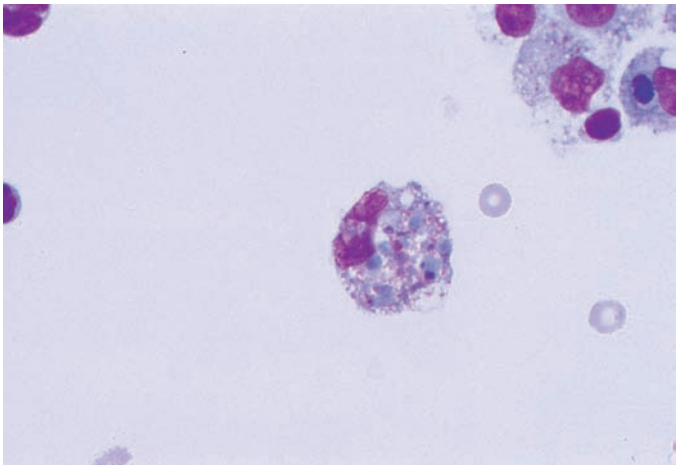


FIGURE 30-14 Macrophage in pleural fluid.

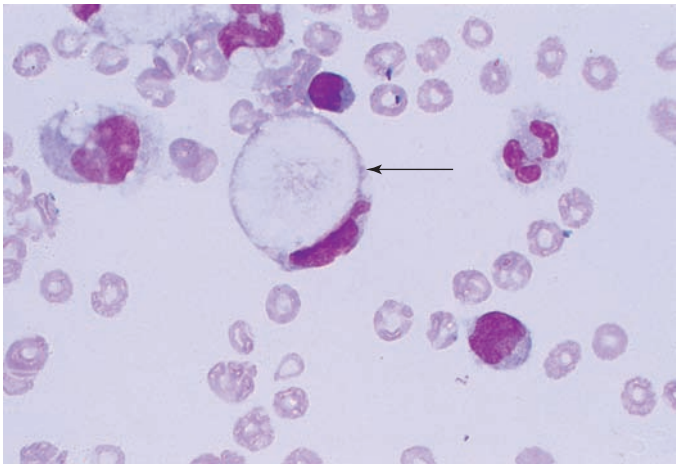


FIGURE 30-15 Macrophage in pleural fluid with single large vacuole giving a "signet ring" appearance.

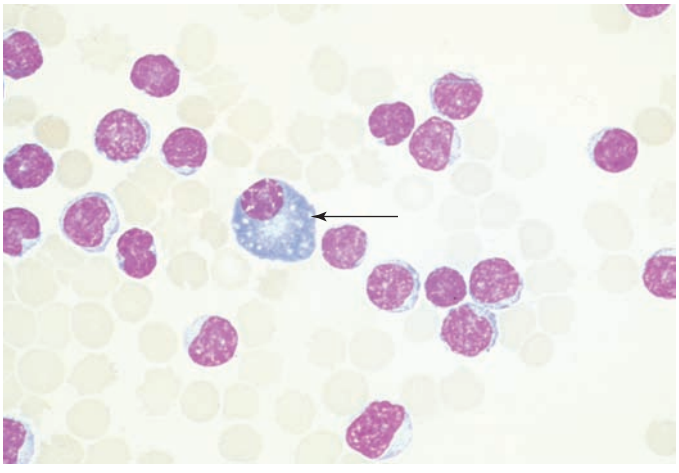
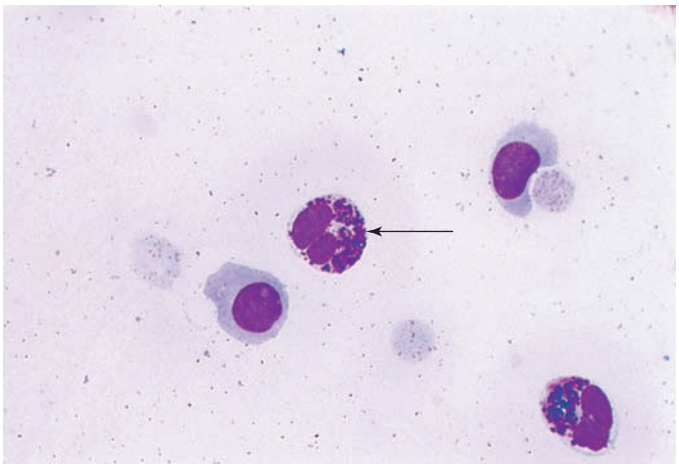
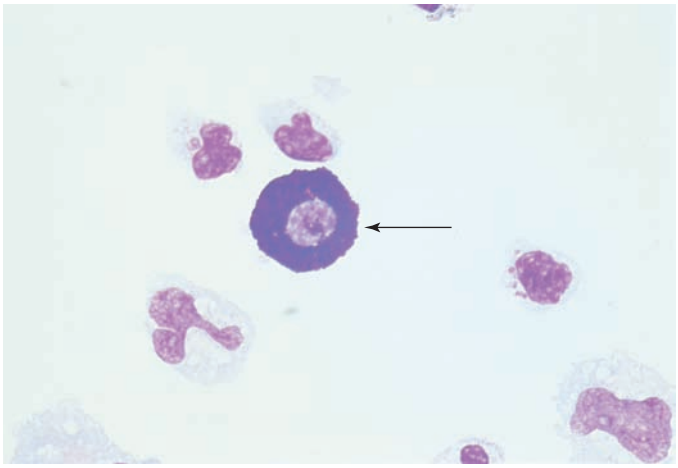


FIGURE 30-16 Plasma cell in pleural fluid.



■ **FIGURE 30-17** Basophil (arrow) in pleural fluid.



■ **FIGURE 30-18** Mast cell (arrow) in pleural fluid.

number of basophils can correlate with myeloproliferative disorders involving body fluids.

Benign tissue cells can be seen in any of the body fluids and must be differentiated from malignant cells (Table 30-5 ★). The morphology of these tissue cells varies according to the fluid space and is discussed in the "Microscopic Findings in Specific Fluids" section.

When performing the nucleated cell differential on body fluids, all cell types should be differentiated if possible. Abnormal or reactive cells that cannot clearly be assigned to a specific category should be counted as unclassified cells and described. Clumps of cells (benign or malignant) should be noted because they can adversely affect the count's accuracy. A comment such as "cells present in clumps, count could be falsely decreased" should be reported.

Morphologic Findings Resulting from Artifact

Some artifactual findings such as peripheral displacement of the nucleus in neutrophils, lymphocyte cytoplasmic extensions, and overly prominent nucleoli of lymphocytes have already been mentioned (Table 30-6 ★). Other artifactual changes can resemble actual pathologic findings, so interpretation must be made cautiously. Starch particles can be an in vitro contaminant in any type of body fluid. Although most surgical gloves in use are now powder free, starch can be on sterilized surgical gloves used by the physician obtaining fluid from the patient. Starch particles can look like yeast organisms, even budding yeasts if two particles are closely associated. Starch particles usually have a refractile center that is not a characteristic feature of yeast. They are also birefringent (discussed in the section "Joint Fluid"), showing as bright Maltese crosslike figures with polarized light (Figure 30-19 ■). Precipitated stain can look like bacterial organisms. If the precipitate appears to be intracellular, changing the fine focus usually reveals the precipitate to be in a different plane of focus than the cell. True intracellular bacteria are in the same plane of focus as the cell. Stain precipitate is darker than bacteria and variable in size and can be seen extracellularly, sometimes in distant areas of the slide (Figure 30-20 ■). In difficult cases, an extra slide should be prepared for a gram stain. The cytocentrifuge exaggerates early cellular degeneration, and cellular nuclei show irregular nuclear margins and separating chromatin. This can be mistaken for malignant cells (Figure 30-21 ■).

Nonspecific Reactive Changes

The term *nonspecific reactive changes* refers to effusions that have an inflammatory cell response that is not diagnostic for any specific disorder. In various pathologic states, certain types of WBCs can be present in increased numbers. Bacterial infections have a predominance of segmented neutrophils, whereas viral, fungal, and mycobacterial infections can have a predominance of lymphocytes or show a mixed inflammatory response.

★ **TABLE 30-5** Normal Existing Tissue Cells Found in Various Body Fluids

Fluid Type	Normal Tissue Cells
• Cerebrospinal	Ependymal, choroid plexus cells, arachnoid
• Pleural, pericardial, peritoneal	Mesothelial
• Joint	Synovial
• Broncoalveolar lavage	Bronchial

★ **TABLE 30-6** Artifacts That Can Be Seen with Wright-Stained Cytocentrifuge Prepared Slides and Potential Mistaken Interpretation

Artifact	Mistaken Interpretation
• Peripheral localization of nuclear lobes in neutrophils	Hypersegmented neutrophils
• Degenerating neutrophil	Yeast organisms, nucleated RBCs
• Overprominence of nucleoli in lymphocytes	Blast cells
• Cytoplasmic projections of lymphocytes	Hairy cell leukemia
• Single large vacuole in histiocyte	Signet ring cell carcinoma
• Starch particles	Yeast organisms, crystals
• Stain precipitate	Bacterial organisms
• Degenerating tissue cells	Malignant cells

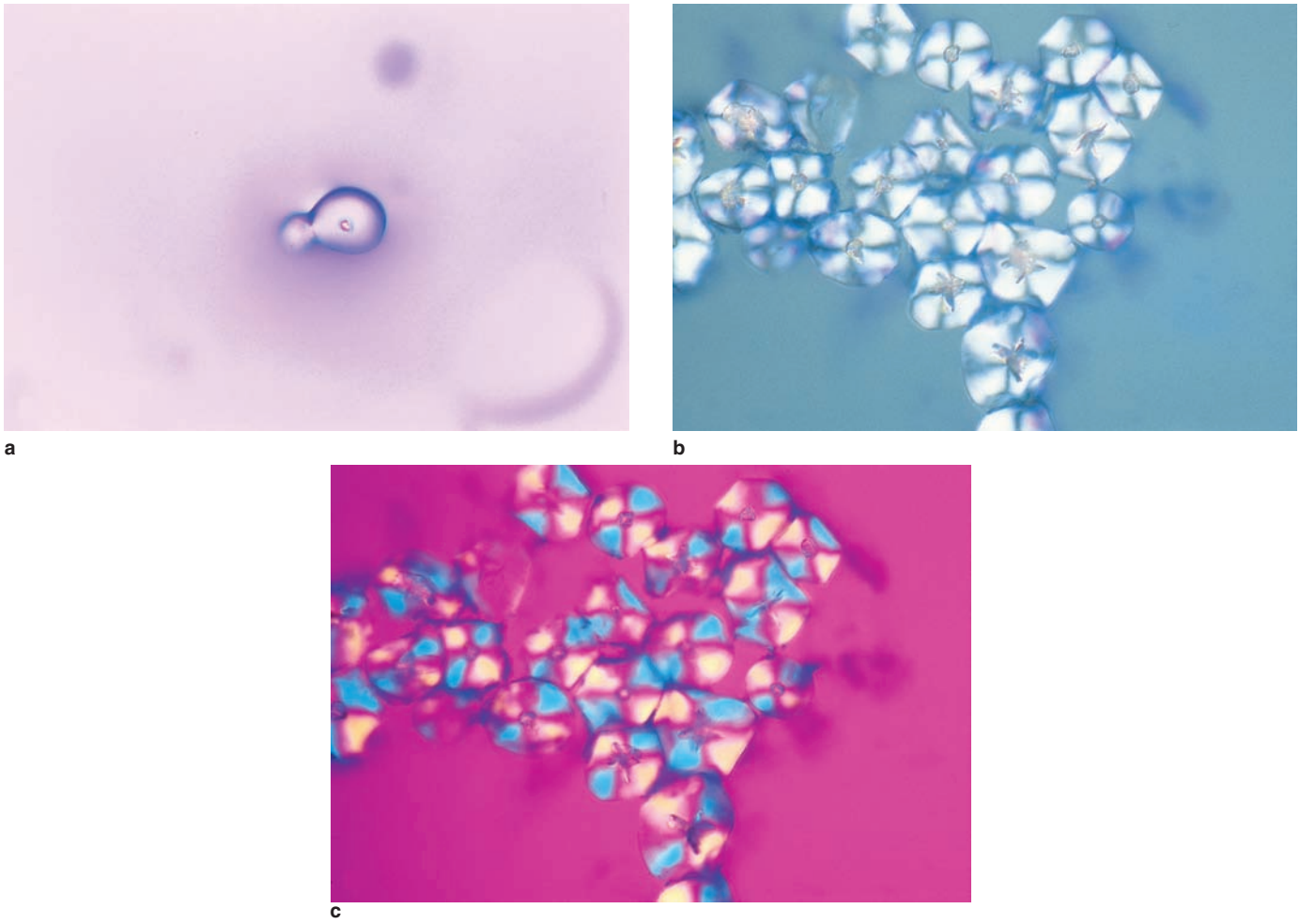


FIGURE 30-19 **(a)** Starch particles with plain light resembling yeast. **(b)** Starch particles as seen with polarized light appearing as Maltese cross shape. **(c)** Starch particles with polarized light and quartz compensator.

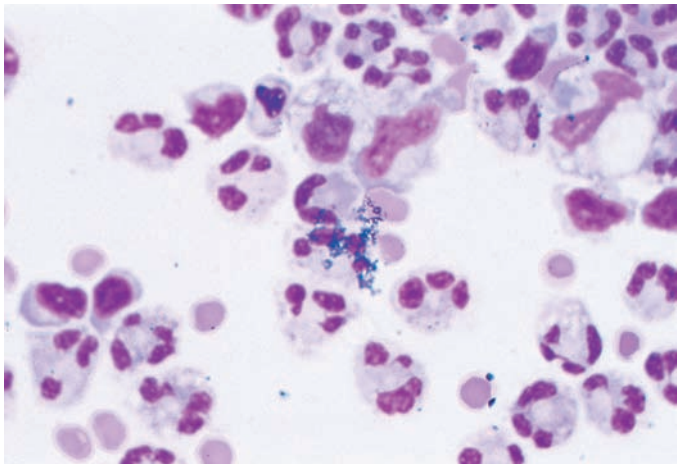


FIGURE 30-20 Stain precipitate on top of the cell with precipitate in focus and cells slightly out of focus.

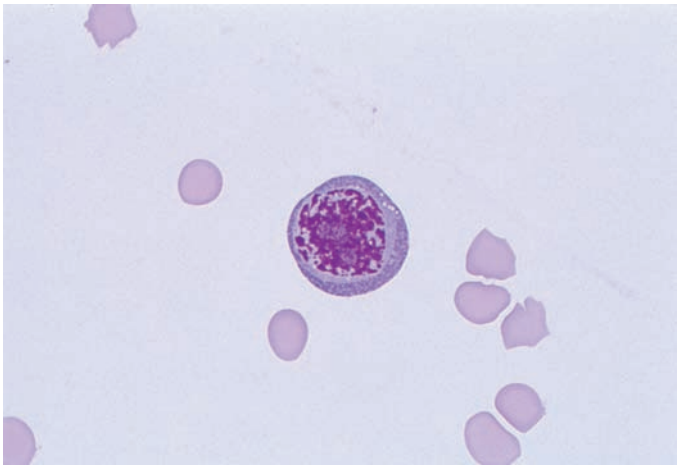
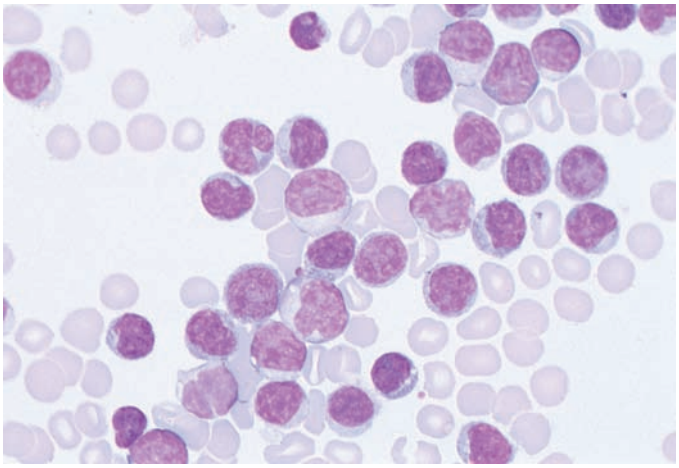
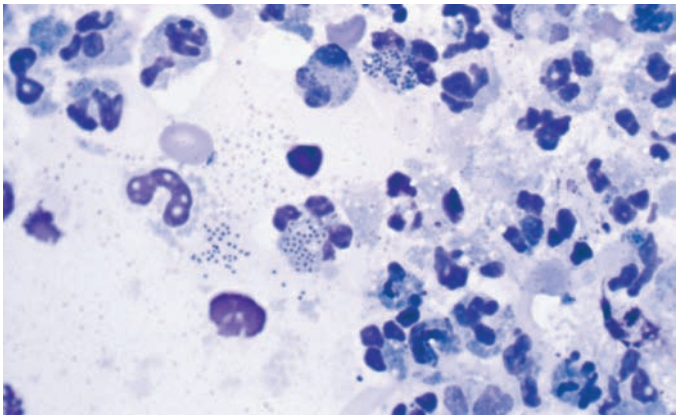


FIGURE 30-21 Early cell degeneration.



■ **FIGURE 30-22** Reactive lymphocytes in pleural fluid.



■ **FIGURE 30-24** Intracellular and extracellular bacterial cocci in joint fluid.

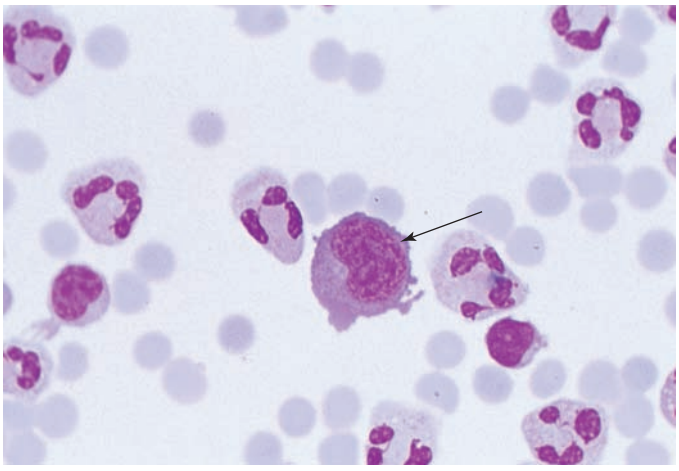
As in peripheral blood, neutrophils can have toxic granulation, Döhle bodies, and cytoplasmic vacuoles (Chapter 21).

Lymphocytes are frequently reactive and transformed, simulating lymphoma cells (Chapters 22, 28). The most helpful feature in distinguishing reactive lymphocytes from lymphoma cells is that the former consist of a heterogeneous population of cells with varying nuclear shape, amount of cytoplasm, and degree of cytoplasmic basophilia (Figures 30-22 ■ and 30-23 ■). Lymphoma cells are homogeneous with the same nuclear and cytoplasmic features. The morphology of the lymphoma cells depends on the particular type of lymphoma.

Microorganisms

Most types of pathogenic bacterial and fungal organisms stain with Wright stain and are detectable on a routine cytocentrifuge preparation. Bacteria stain blue regardless of the Gram stain features. Recognizing the presence of intracellular organisms is important because it indicates true pathogenicity rather than in vitro contamination (Figure 30-24 ■ and Web Figure 30-6). Once bacteria have been recognized with Wright stain, preparing a second cytocentrifuge slide for a Gram stain is helpful to confirm the presence of bacteria and to give additional information to the physician while cultures are pending. Caution must be used when suspected bacteria are seen in synovial fluids treated with hyaluronidase. Hyaluronidase products can be labeled as sterile, but on rare occasions can contain nonviable bacteria. Thus, the cytocentrifuge slide made for a confirmation Gram stain should be prepared using the untreated fluid. Likewise, the albumin used to provide additional protection and cell stability when making cytocentrifuge slides of serous and cerebral spinal fluids can be a source of bacterial contamination.

Most pathogenic yeasts are found in CSF rather than in pleural, pericardial, or peritoneal fluids, which can be found intracellularly. The different types of pathogenic yeast show some distinguishing features on Wright-stained slides. This morphologic variance can be used as a clue to an initial impression of the specific type of yeast, but cultures must be obtained for definitive identification. The most frequently seen fungal organisms in fluids are *Cryptococcus*, *Histoplasma*, *Candida albicans*, and *Candida tropicalis* (Web Figures 30-7 through 30-10). Refer to Web Table 30-4 for a comparison of morphology.



■ **FIGURE 30-23** Reactive lymphocyte (arrow) in pleural fluid.

Malignant Cells in Body Fluids

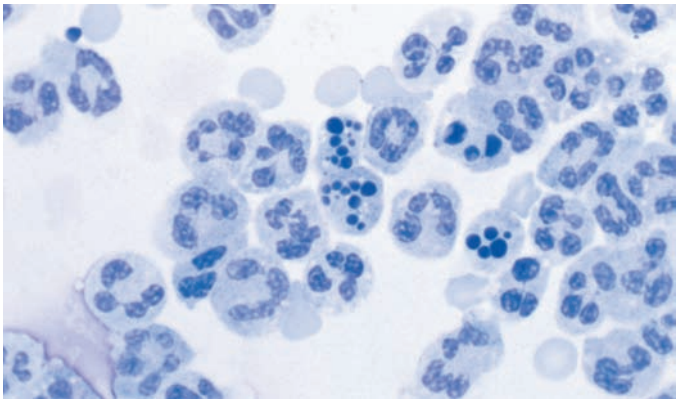
Malignant cells can be seen in any fluid but are found more often in serous fluids. They are rare in synovial fluid. Malignant cells in serous fluids pose the greatest difficulty for the laboratory because they are often difficult to differentiate from mesothelial cells. Differentiating characteristics are discussed in the section "Microscopic Findings in Specific Fluids."

In some patients, a diagnosis of malignancy already can have been established by other tissue sampling (biopsy or excision), and finding the malignant cells in fluid establishes a condition of tumor metastasis. For other patients, finding malignant cells in fluid can be the initial diagnosis of a malignancy, and if a sample has not been sent to cytology, the recognition of malignancy by the hematology laboratory is critical in establishing an early diagnosis. Malignant cells in fluids can usually be distinguished as hematopoietic in origin (leukemia, lymphoma) versus nonhematopoietic (carcinoma,

CASE STUDY *(continued from page 579)*

A cytocentrifuged, Wright-stained slide is prepared and a photomicrograph is taken (Figure 30-25 ■).

3. What is an appropriate next step to determine whether the material seen is debris or true organisms?
4. Some of the large tissue cells showed features of degeneration and suspicious for malignancy. How should this be interpreted?

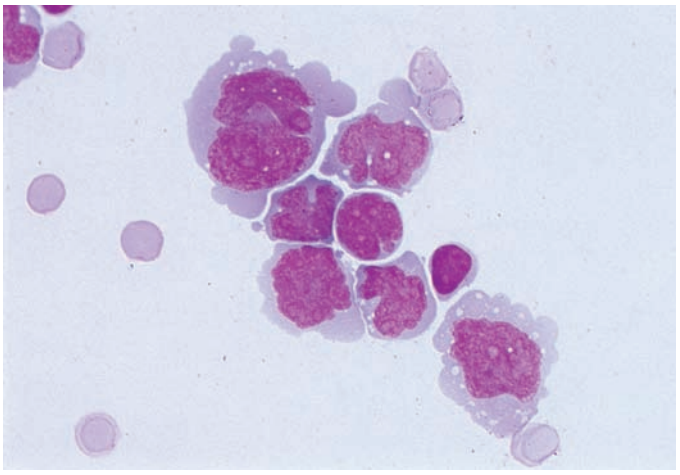


■ **FIGURE 30-25** Pleural fluid.

sarcoma), and in some cases, additional specification of cell type is also possible. It is important to look at the entire cellular area of the slide with a low power objective (10×) to detect suspicious clusters of cells.

Almost any type of hematopoietic malignancy, including lymphocytic and nonlymphocytic leukemias, lymphomas, Hodgkin disease, and plasma cell neoplasms,[27] can be found in body fluids. Generally, the abnormal cells found in the body fluids in these disorders have the same morphologic features as peripheral blood and bone marrow cells. The acute leukemias only occasionally involve the pleural, pericardial, or peritoneal cavities but more often are seen in the CSF. Blasts appear larger on cytocentrifuge preparations than on peripheral blood smears, and the nuclear membrane can be surprisingly irregular. Auer rods can be seen, and, if necessary, unstained slides can be prepared for cytochemistry stains and terminal deoxynucleotidyl transferase (TdT) to differentiate the blasts (Chapters 23, 37). Lymphoblasts have a very high nuclear-cytoplasmic ratio, and the nucleus can be folded or convoluted.

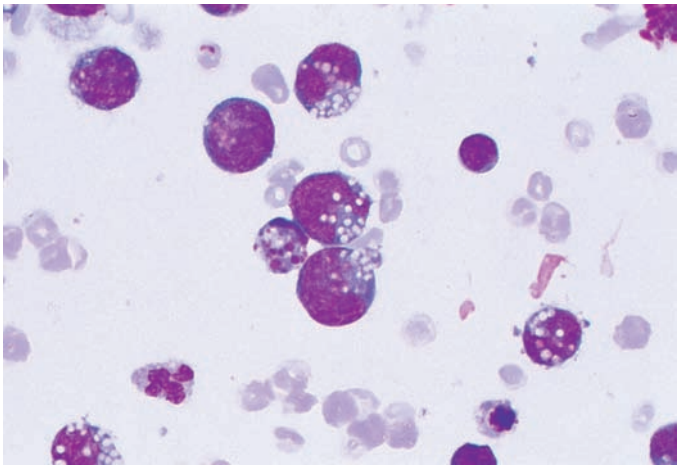
The morphology of non-Hodgkin lymphoma in the body fluids depends on the particular type of lymphoma. Again, the nuclear membrane can be surprisingly irregular. Large-cell lymphoma has cells that are moderate to large in size with irregular nuclei, partially clumped chromatin, and sometimes prominent nucleoli (Figure 30-26 ■). The cytoplasm is low to moderate in amount and



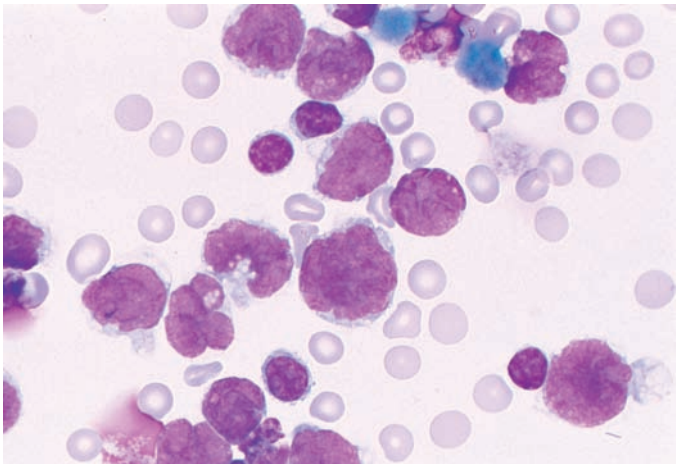
■ **FIGURE 30-26** Large-cell lymphoma in pleural fluid.

basophilic. The cells are discohesive (a loosely joined cluster), but if the fluid is very cellular, the cells can be thrown together and have the appearance of carcinoma cell clusters; nuclear molding is not seen. Burkitt lymphoma has intermediate size cells with more than one nucleoli and an immature blastlike chromatin (Figure 30-27 ■). Prominent cytoplasmic vacuoles frequently are apparent. Small-cell lymphoma is the most difficult to diagnose and can look like a benign lymphocytic infiltrate. In these cases, flow cytometry is valuable in demonstrating a clonal population of cells (Chapter 40). T-cell lymphoma can show markedly irregular, convoluted nuclei; however, marker studies are necessary to confirm the T- or B-cell origin of the neoplastic cells (Figures 30-28 ■ and 30-29 ■). Cell origin identification can be accomplished by flow cytometry or immunoperoxidase techniques on cytocentrifuge-prepared slides.

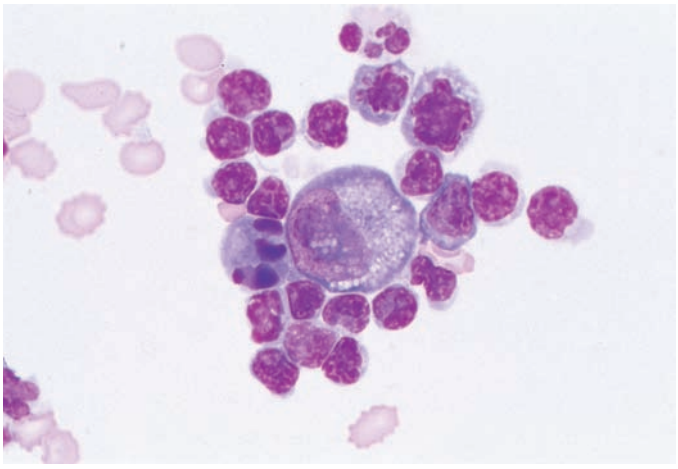
Primary effusion (body cavity) lymphoma is a high-grade disease found only in a body cavity without an associated solid tumor mass. This unique malignancy has been reported in patients who are



■ **FIGURE 30-27** Burkitt lymphoma (small noncleaved cell) in pleural fluid.



■ **FIGURE 30-28** Lymphoblastic lymphoma, T-cell type, in pleural fluid.



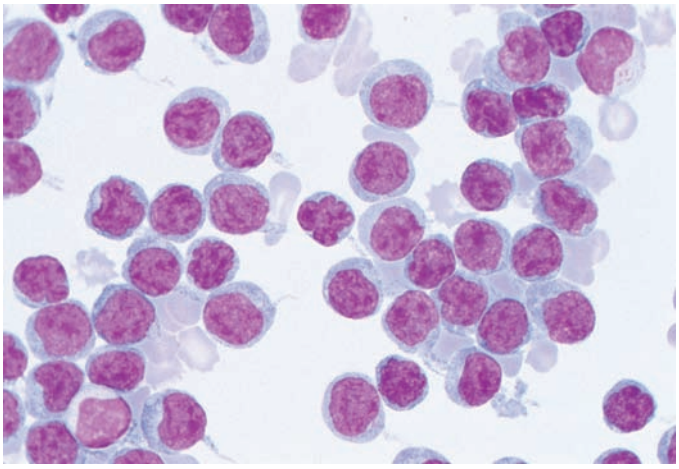
■ **FIGURE 30-30** Hodgkin disease, pleural fluid.

☑ CHECKPOINT 30-2

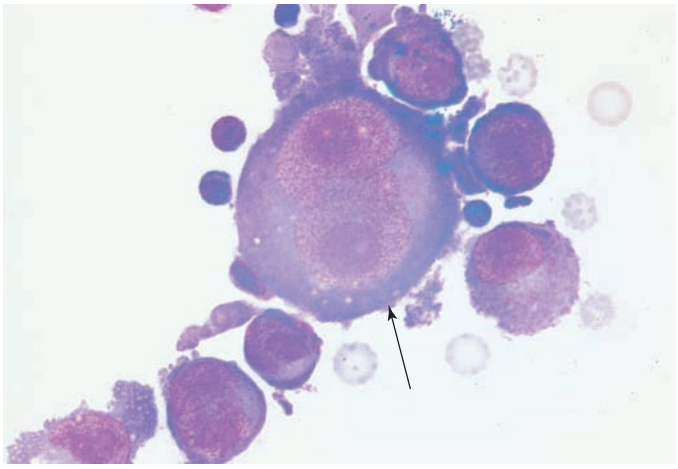
A 32-year-old woman has right-side chest pain and shortness of breath that has worsened over a two-week period. Chest radiologic studies reveal a right pleural effusion, and a thoracentesis is performed. The pleural fluid specimen on a cytocentrifuged, Wright-stained slide reveals cells similar to that seen in Figure 30-10. What is the best interpretation of this finding? If there is a strong concern that this can represent a low-grade lymphoma, what would be the best way to determine whether these are benign or malignant lymphocytes?

immunocompromised, usually HIV positive, and is associated with human Herpes virus 8 (HHV-8; also known as *Kaposi sarcoma*–associated herpes virus, KSHV).[28–30] The morphology of the cells in the effusion is similar to the morphology of cells found in anaplastic large-cell lymphoma, diffuse large B-cell lymphoma, or small lymphocytic lymphoma. These malignant cells are usually B-cell derived but lack surface-associated antigens for T- or B-cell lineage.

Hodgkin lymphoma can occasionally be seen to involve pleural fluid (Figures 30-30 ■ and 30-31 ■). The malignant Hodgkin cell is large with a moderate to abundant amount of cytoplasm, large nuclei, and prominent nucleoli. If the nucleus is bilobed or if the cell has two nuclei, it can be a Reed-Sternberg cell. The other cells present consist of varying numbers of small lymphocytes, eosinophils, histiocytes, and plasma cells. If other tissue biopsy has already established a patient's Hodgkin disease diagnosis, the malignant cells (either Hodgkin, Reed-Sternberg, or multinucleated variants) must still be identified in the effusion sample to diagnose the fluid's involvement.



■ **FIGURE 30-29** Small lymphocytic lymphoma.

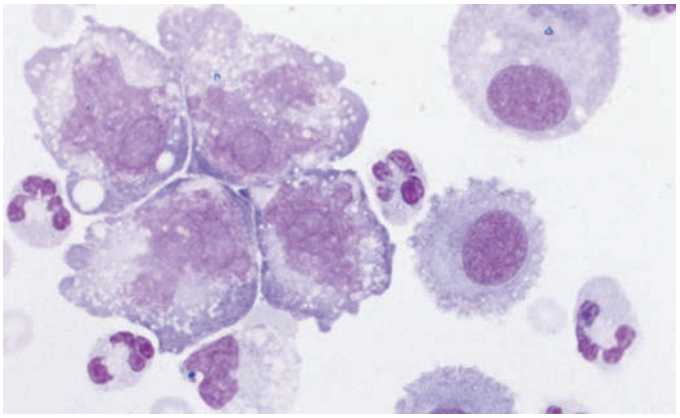


■ **FIGURE 30-31** Hodgkin disease with Reed-Sternberg cell (arrow), pleural fluid.

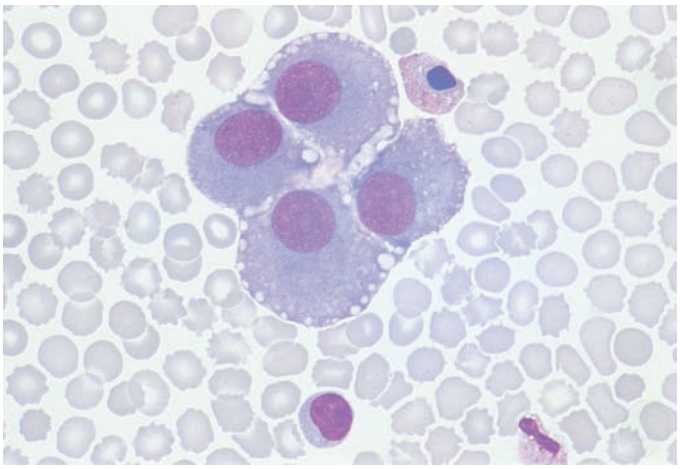
CASE STUDY (continued from page 588)

After 3 weeks, Carolyn improved significantly, but the chest pain and effusion did not resolve. A repeat thoracentesis was performed. Laboratory studies show protein 4.7 g/dL (serum = 60 g/dL), lactate dehydrogenase 50 U/L (serum = 60 U/L), and total nucleated cell count 3,000/mcL. A cytocentrifuged, Wright-stained slide is examined, and the differential count shows 30% segmented neutrophils, 20% lymphocytes, 10% histiocytes, and 40% tissue cells. See Figure 30-32 ■ for a photomicrograph of the tissue cells.

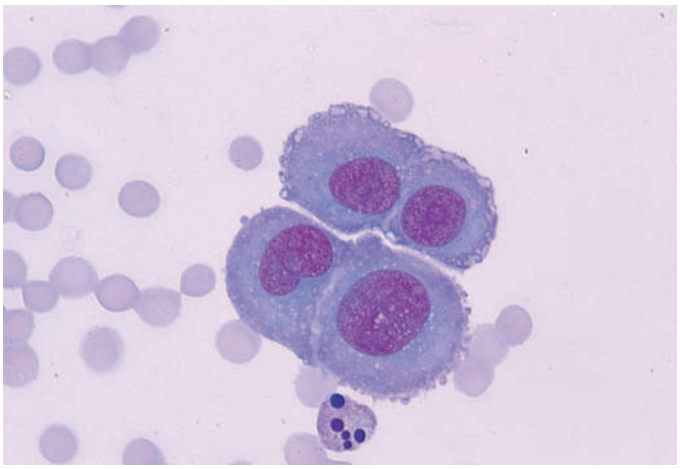
5. Is this an exudate or transudate?
6. What is the most appropriate interpretation of these findings?



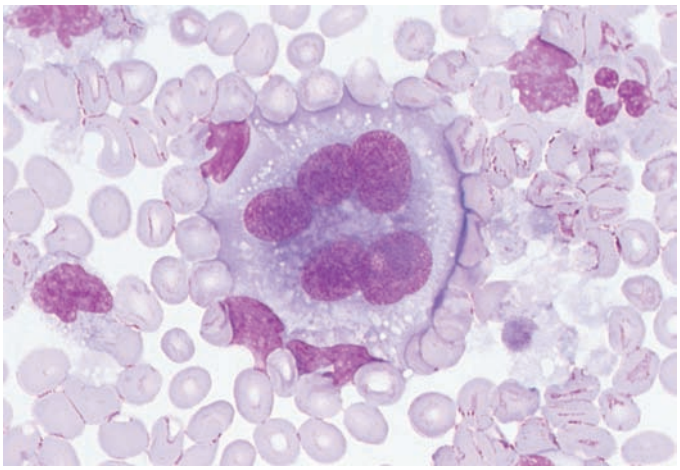
■ **FIGURE 30-32** Pleural fluid.



■ **FIGURE 30-33** Benign mesothelial cells in pleural fluid.



■ **FIGURE 30-34** Reactive mesothelial cells in pleural fluid.



■ **FIGURE 30-35** Multinucleated mesothelial cell in pleural fluid.

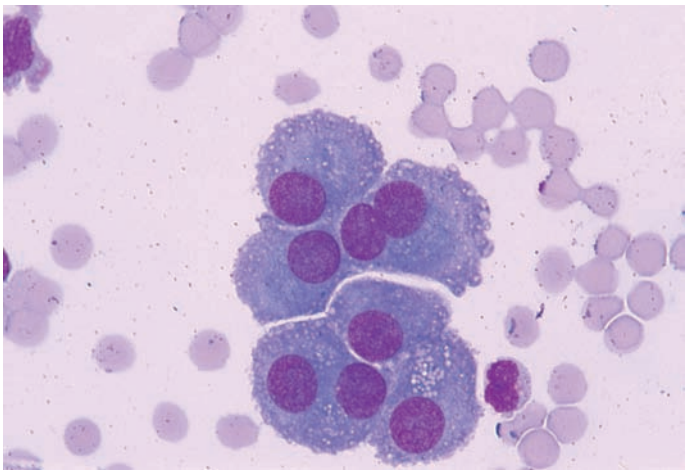
Microscopic Findings in Specific Fluids

Serous: Pleural, Peritoneal, Pericardial

Benign mesothelial cells can be seen in the pleural, pericardial, and peritoneal fluids. These are large cells that have a moderate to abundant amount of cytoplasm. The cytoplasm can be light or dark blue and occasionally contain granules or phagocytosed debris. The nucleus is eccentric with a homogeneous chromatin pattern. Nucleoli can be seen, and if present, are blue with a smooth membrane (Figure 30-33 ■).

The pleural, pericardial, and peritoneal fluids also can contain malignant cells, and their identification is critical for making an accurate diagnosis.[31–33] Any one sample can have only a few malignant cells that are difficult to find. Mesothelial cells can show nonspecific reactive changes, which include multinuclearity, presence of nucleoli, mitotic activity, basophilic staining, and sometimes an increase in cell size (Figures 30-34 ■ and 30-35 ■). Occasionally there also is an increased nuclear-cytoplasmic ratio and nuclear folding simulating carcinoma.

Reactive mesothelial and malignant cells must be differentiated. Reactive mesothelial cells can tend to cluster and appear cohesive but the clusters appear flat rather than the ball-like clusters of malignant cells. Because reactive mesothelial cells are often seen in clusters, it can be challenging to differentiate the benign and malignant cells in pleural and peritoneal fluids. A useful feature of mesothelial cells is their overall uniformity when viewed under low



■ **FIGURE 30-36** Reactive mesothelial cells in pleural fluid.

power. The round-to-oval nuclei are uniform in appearance as is the generally low nuclear-to-cytoplasmic (N:C) ratio. Clusters of mesothelial cells are flat and the individual cells can be distinguished often showing a clear separation or "window" separating the cells in the cluster (Figure 30-36 ■). In cases when distinguishing reactive mesothelial cells from malignant cells is difficult, cytology preparations usually are definitive because alcohol fixation and Papanicolaou stain yield better nuclear detail. As a general rule, malignant cells do not have this uniform appearance, and the nuclear shapes and sizes can vary dramatically. The N:C ratio is often high, and clusters of cells can be ball-like, making distinguishing the individual cells difficult.

General features that can be seen in almost any type of malignant cell include an irregular nuclear membrane, unevenly distributed chromatin, and nucleoli that also have an irregular membrane (Table 30-7 ★).[32] The N:C ratio varies with small carcinoma cells having minimal cytoplasm and adenocarcinoma cells having as much or more cytoplasm as a benign mesothelial cell. The nuclear membrane irregularity can be jagged or have multiple folds. When nucleoli are present, they are frequently prominent and irregular in shape (Figure 30-37 ■). Mitotic activity alone is not a reliable indication of malignancy because reactive mesothelial cells can undergo mitosis. Nor are cytoplasmic vacuoles a reliable finding for malignancy because their presence can be part of early degeneration in many cells. None of the features described can be used alone to diagnose malignancy. All of the features must be looked for and evaluated together. For example, one type of malignancy can show smooth nuclear membranes but with unevenly distributed chromatin and irregular nucleoli (Table 30-8 ★).

The most common nonhematopoietic malignancies seen in body fluids are small-cell carcinoma and adenocarcinoma. Small-cell carcinoma cells have the same general morphologic findings of malignant cells but can be distinguished from other types of carcinoma cells because of the characteristic high N:C ratio, blastlike chromatin, absence of nucleoli or nonprominent nucleoli, and frequent nuclear molding (the process of the nucleus of one cell molding around the shape of an adjacent cell) (Figure 30-38 ■). Nuclear molding occurs with cohesive growth of cells requiring the presence of tight junctions

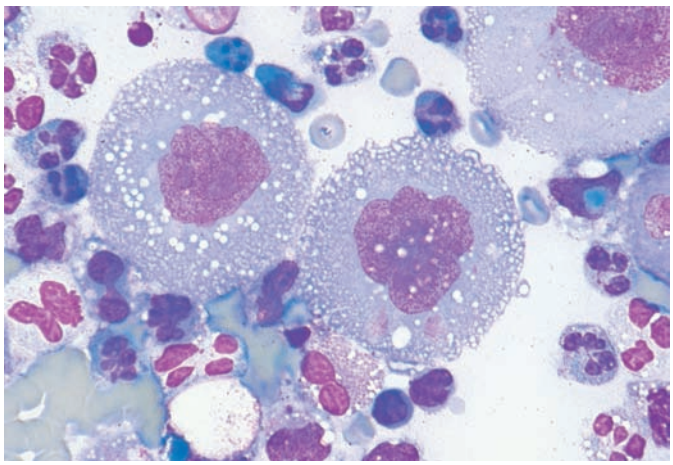
★ **TABLE 30-7** Comparison of Morphologic Features of Reactive Mesothelial Cells versus Malignant Cells

Cell Features	Reactive Mesothelial Cells	Malignant Cells
Nuclear membrane	Smooth	Irregular, jagged
Chromatin	Evenly distributed	Unevenly distributed
Nucleoli	Absent or present with smooth membrane	Prominent, frequently multiple, irregular membrane
Nuclear molding	None	Present in nonhematopoietic malignancies

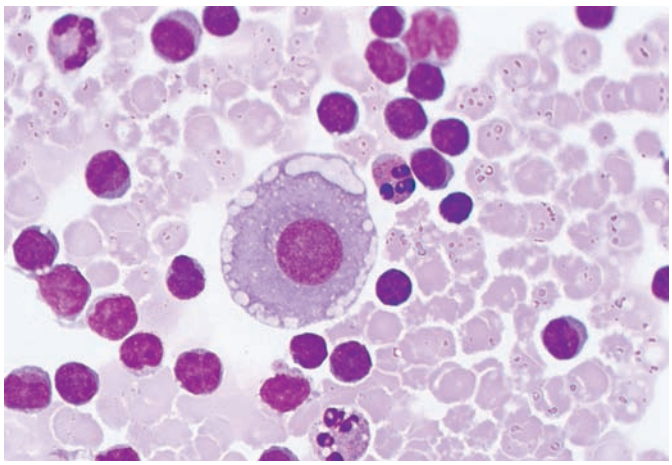
★ **TABLE 30-8** General Morphologic Findings of Benign Mesothelial Cells and Malignant Cells[a]

	Mesothelial Cell	Adenocarcinoma	Small-Cell Carcinoma	Large-Cell Lymphoma	Leukemic Blasts
Cell size	Large, 15–30 mcM	Large to giant	Moderate to large	Moderate to large	Small to moderate
Chromatin	Loose, homogeneous	Partially clumped, heterogeneous	Slightly course, homogeneous	Partially clumped, heterogeneous	Smooth, lacelike, homogeneous
Nucleoli	None to small and regular	Prominent, multiple, irregular	None to small, not prominent	Small to prominent, irregular	Variable
Nuclear membrane	Smooth	Irregular, jagged	Irregular, jagged, folded	Irregular, jagged, folded	Smooth or irregular, folded
N:C ratio	Low, 1:3–5	Low, 1:3 or less	High, 1:1.25	High to moderate 1:1.25–1:2	High to moderate 1:1.25–1:1.75
Intercellular relationship	Individual or clumped, no nuclear molding	Usually clumped ± nuclear molding	Clumped with nuclear molding, occasionally individual	Individual, no clumping, no nuclear molding	Individual, no clumping, no nuclear molding

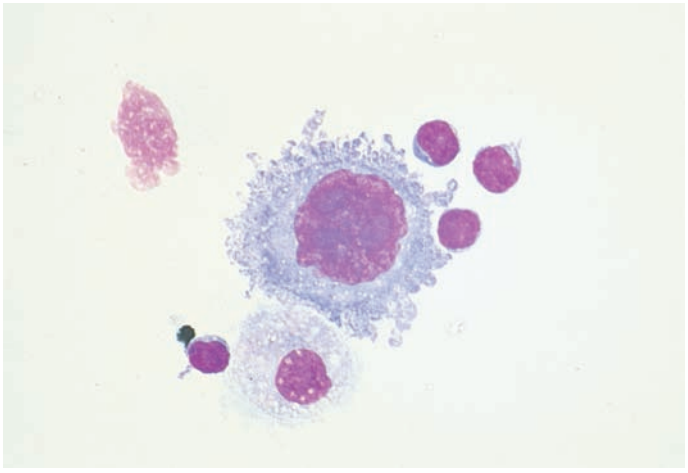
[a]Any given cell can show variable features, so all must be evaluated before deciding whether a body fluid sample is benign or malignant. No single feature can be used to diagnose malignancy.



a

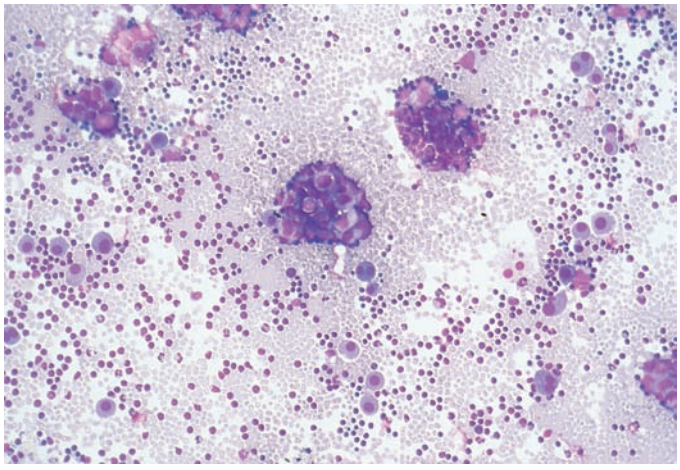


b

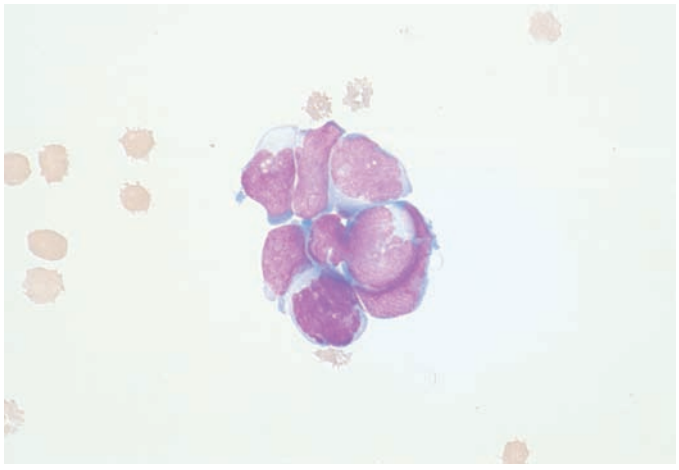


c

FIGURE 30-37 **(a)** Malignant cells (adenocarcinoma), pleural fluid. **(b)** Benign mesothelial cell to contrast with features of malignant cell. **(c)** Single malignant cell (adenocarcinoma), pleural fluid.

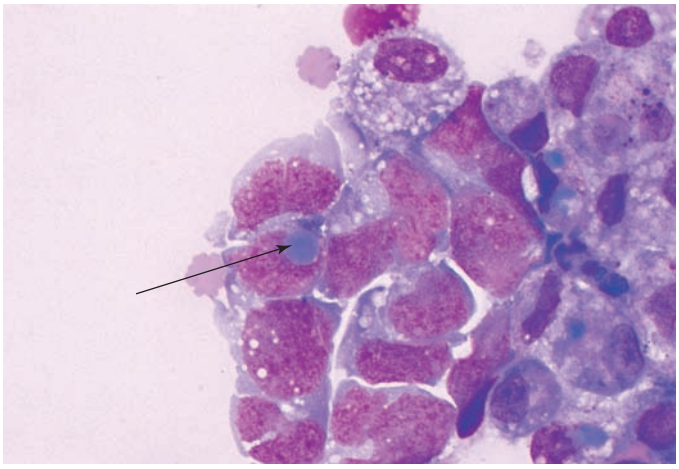


a



b

FIGURE 30-38 **(a)** Small-cell carcinoma, pleural fluid showing tight cell clusters (original magnification, 25×). **(b)** Small-cell carcinoma.



■ **FIGURE 30-39** Small-cell carcinoma, paranuclear "blue body" (arrow) in malignant cell.

between the cytoplasmic membranes of the cells. Therefore, nuclear molding can be seen in any type of carcinoma but is most often seen with small-cell carcinoma. Some cells also can have a paranuclear "blue body," which is an inclusion that has not yet been characterized and can represent early cell degeneration or phagocytosed material. Depending on the cell's orientation, the blue body can appear to be intranuclear and has been described in small-cell carcinoma but rarely in sarcoma.[34] The blue body is seen only with air-dried, Wright-stained preparations (Figure 30-39 ■). If the malignant cells are noncohesive, small-cell carcinoma could be mistaken for a hematopoietic malignancy, and finding a blue body would be a good clue for the diagnosis of small-cell carcinoma.

Adenocarcinoma differs from small-cell carcinoma in that the overall size of an adenocarcinoma cell is larger than a small-cell carcinoma cell with a moderate to abundant amount of cytoplasm (Figure 30-40 ■). The nuclear chromatin is partially clumped and heterogeneous and has prominent nucleoli. The presence of cytoplasmic vacuoles is not specific and can represent early cell degeneration.

Other types of carcinoma and sarcoma can be found in body fluids (Web Figures 30-11 through 30-14). The features seen with Wright stain are not as specific as a cytology preparation; the latter is necessary to specifically identify the type of malignancy. For example, squamous cell carcinoma can look like adenocarcinoma with Wright stain; however, in most cases, the two are readily distinguishable on cytology preparations (Web Figure 30-15). Certain types of malignant cells can contain clues to their cellular origin. Melanoma cells can have melanin pigment that is demonstrable with Wright stain, and hepatocellular carcinoma can have bile pigment (Web Figures 30-16 and 30-17). The presence of these pigments can be suspected with Wright stain but must be confirmed with more specific staining techniques.

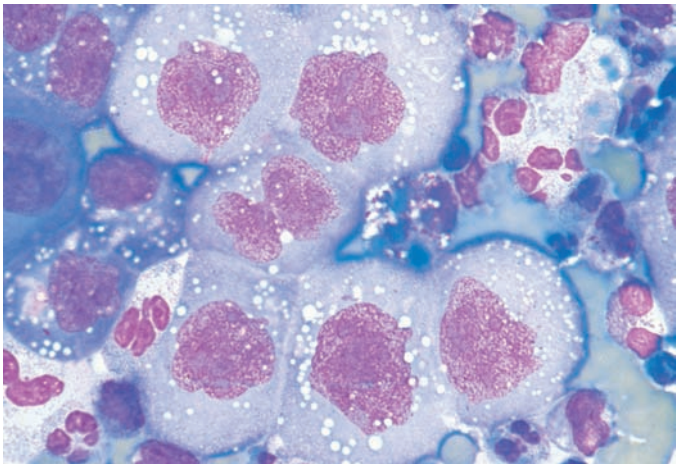
☑ CHECKPOINT 30-3

What are the best features to use in determining whether tissue cells are benign or malignant when examining a cytocentrifuged, Wright-stained slide of a body fluid specimen?

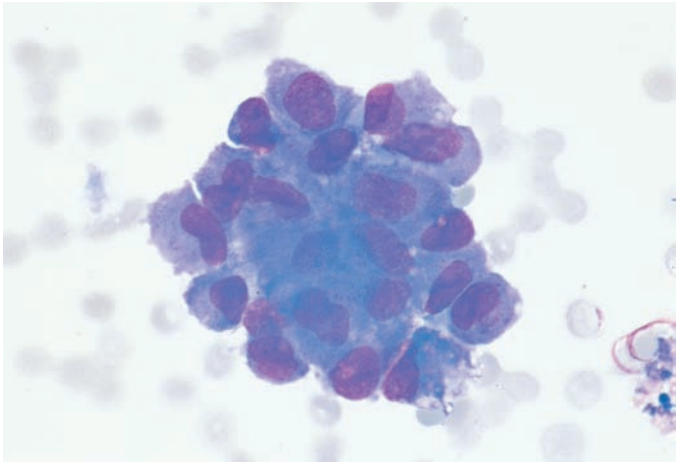
In rare cases, spontaneous formation of lupus erythematosus (LE) cells is seen in serous fluids. An LE cell is a macrophage, either neutrophil or monocyte, that has phagocytosed a nucleus showing a homogeneous, smooth chromatin pattern (Web Figure 30-18). Finding these cells is suspicious but not diagnostic for SLE. Other autoimmune disorders can also show the LE cell phenomenon. Nevertheless, the identification of these cells can be extremely helpful in arriving at a diagnosis difficult to make. The LE cell should not be mistaken for simple phagocytosis of cells by macrophages, which is frequently seen. The chromatin of the usual phagocytosed cell is neither smooth nor homogeneous.

Cerebral Spinal Fluid (CSF)

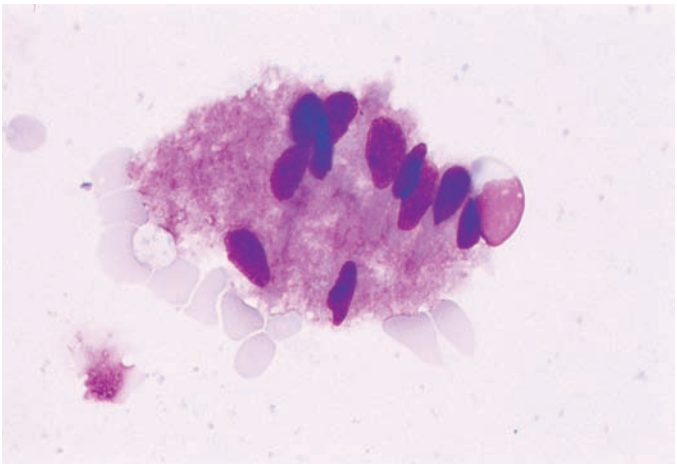
The tissue cells (choroid plexus and ependymal) that can be seen in CSF tend to cluster and can have cytoplasmic granules and slightly irregular nuclei (Figure 30-41 ■). The arachnoid cells are frequently seen as a syncytium with a mass of cytoplasm containing several nuclei (Figure 30-42 ■). These benign tissue cells are usually seen in CSF



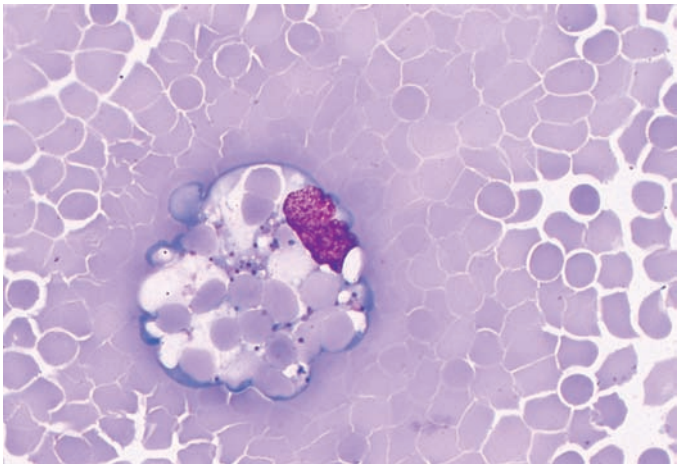
■ **FIGURE 30-40** Adenocarcinoma in pleural fluid.



■ **FIGURE 30-41** Choroid plexus cells in CSF.



■ **FIGURE 30-42** Arachnoid cell syncytium in CSF.



■ **FIGURE 30-44** Erythrophagocytosis in CSF.

only from infants and adults who have had some type of manipulation such as recent neurosurgery or shunt placement. The appearance of these cells from a spinal tap procedure in an adult is very unusual. CSF tissue cells can resemble mesothelial cells, but they are found only in the serous spaces, not in CSF.

As with the pleural, pericardial, and peritoneal fluids, reactive lymphocytes must be distinguished from lymphoma cells (Figure 30-43 ■). Hematopoietic precursors, as well as megakaryocytes, can be present if the spinal tap needle penetrated the vertebral bone, drawing back a portion of bone marrow. This is most often seen in infants but can occur in adults with osteoarthritis.

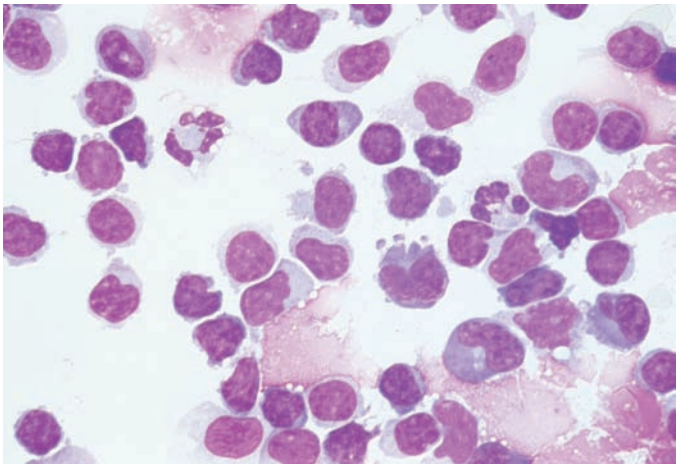
A definitive sign of CNS hemorrhage is phagocytosis of erythrocytes by histiocytes (erythrophagocytosis) (Figure 30-44 ■). It takes approximately 18 hours for histiocytes to mobilize and phagocytose erythrocytes after a hemorrhage; thus, erythrophagocytosis is not an immediate indicator of a current CNS hemorrhage. In older hemorrhages, hemosiderin and hematoidin crystals can be seen intracellularly or extracellularly (Figure 30-45 ■). Hemosiderin and hematoidin are products of hemoglobin catabolism. Erythrophagocytosis and hemosiderin can be seen on other body fluids and should be reported but has more clinical significance in CSF.

☑ CHECKPOINT 30-4

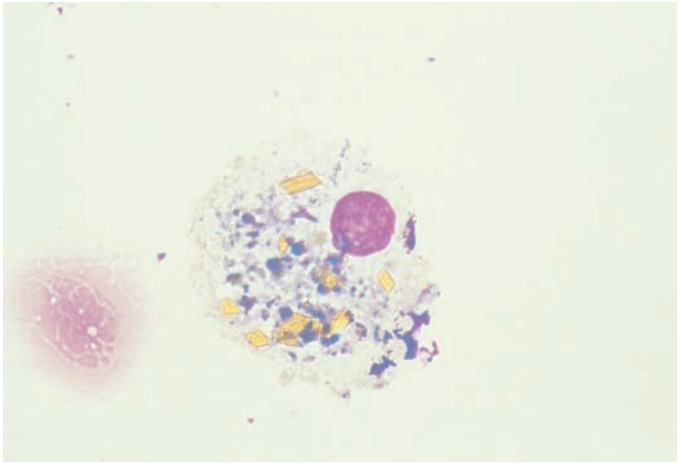
A wife finds her 47-year-old husband comatose at home. During examination in the emergency room, a spinal tap is performed and grossly bloody spinal fluid is obtained. The total RBC count in the first tube is the same as that in the third tube. A cytocentrifuged, Wright-stained slide shows findings similar to that seen in Figure 30-44. What is the most appropriate interpretation of these findings?

Because mesothelial cells are not found in the CSF, the presence of any large tissue cells should be considered suspicious for malignancy (Figures 30-46 ■ and 30-47 ■). Malignant cells, however, must be differentiated from the benign choroid plexus cells, ependymal cells, and arachnoid cells by evaluating them for standard features of malignancy as described earlier. Cytology preparations are usually definitive.

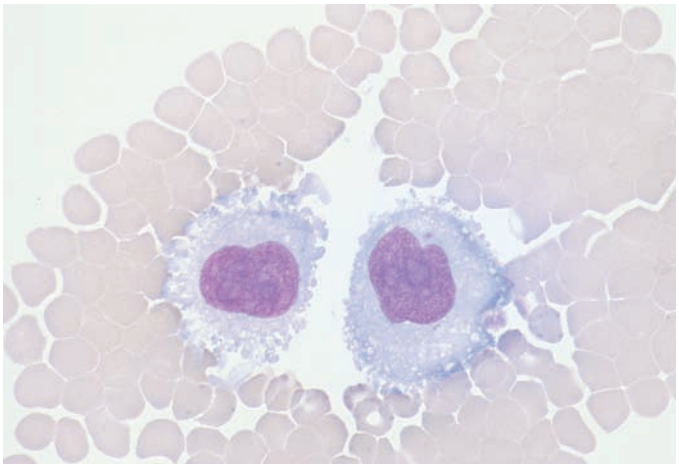
Cells from primary CNS neoplasms rarely are found in the CSF. Medulloblastoma is a malignant tumor usually occurring in



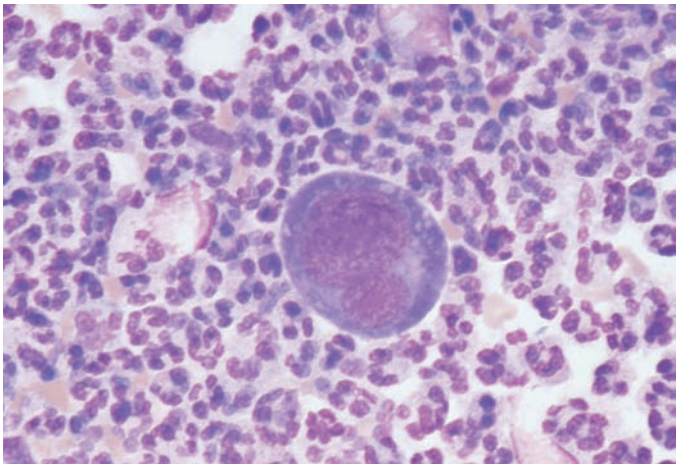
■ **FIGURE 30-43** Reactive lymphocytes in CSF.



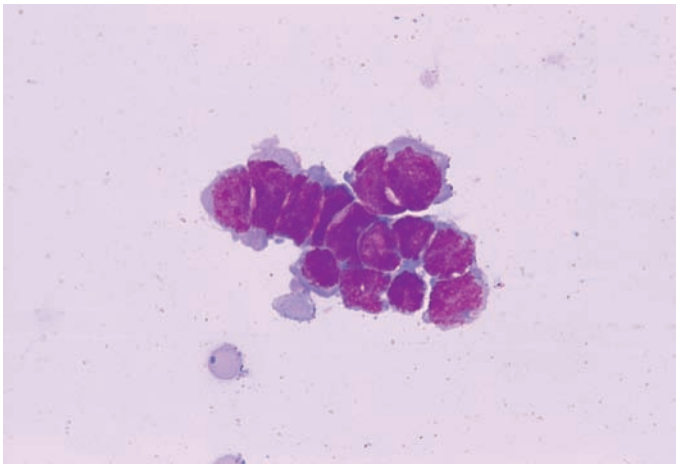
■ **FIGURE 30-45** Hematoidin crystals in macrophage in CSF.



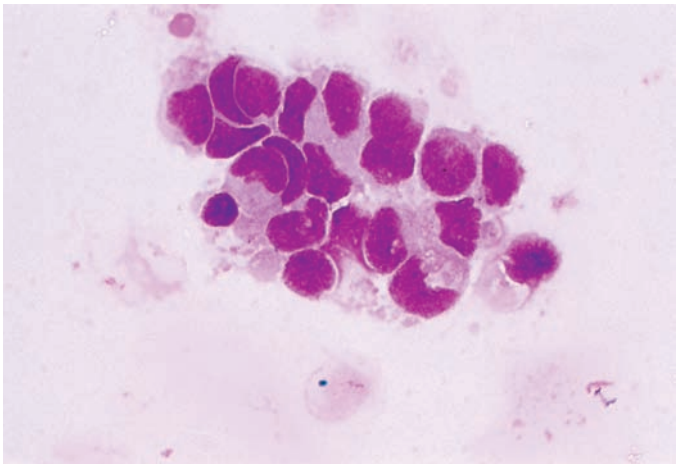
■ **FIGURE 30-46** Adenocarcinoma, CSF.



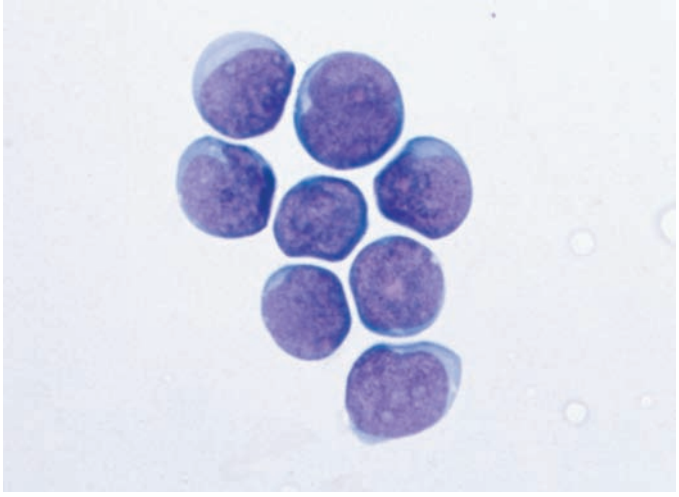
■ **FIGURE 30-47** Large-cell undifferentiated carcinoma with intense chemical acute meningitis, CSF.



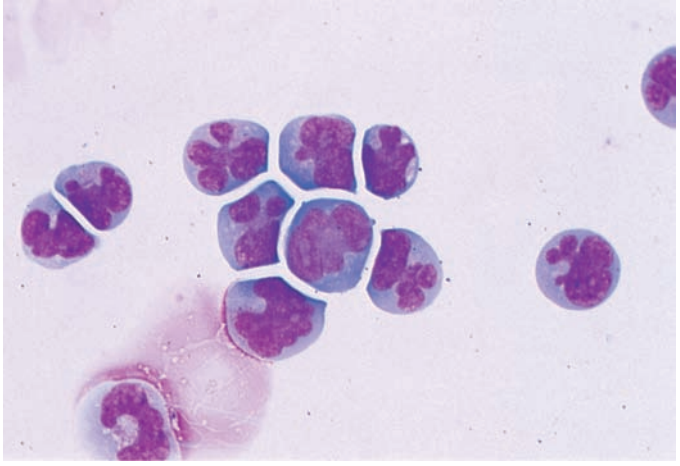
■ **FIGURE 30-48** Small-cell carcinoma, CSF.



■ **FIGURE 30-49** Medulloblastoma, CSF.



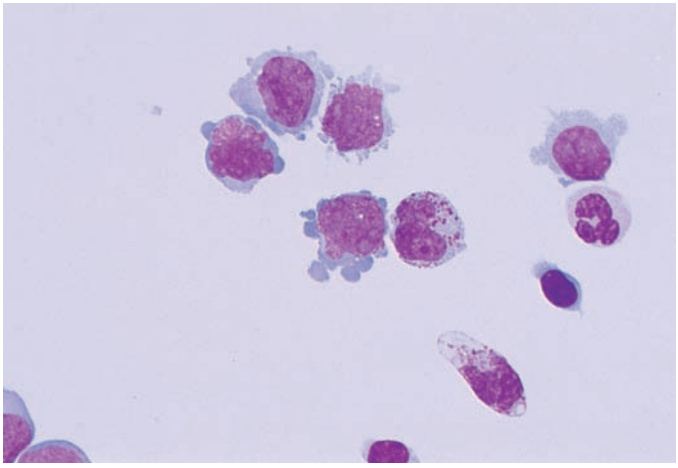
■ **FIGURE 30-50** Acute lymphoblastic leukemia, CSF.



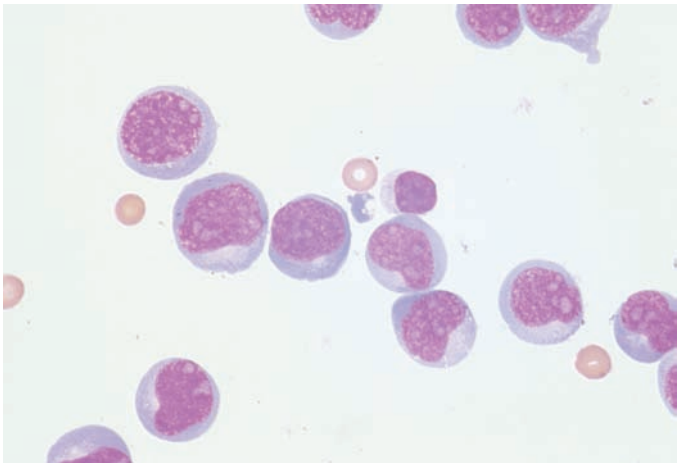
■ **FIGURE 30-51** Acute myelomonocytic leukemia, CSF.

the cerebellum of pediatric patients and has a cellular morphology similar to that of small-cell carcinoma (Figures 30-48 ■ and 30-49 ■). The patient history from the physician is necessary to distinguish the tumor's origin.

Acute lymphoblastic leukemia more often involves the CNS than acute nonlymphocytic leukemia (Figures 30-50 ■ through 30-52 ■). When erythrocytes are present, care must be taken not to overinterpret the presence of blasts that simply represent peripheral



■ **FIGURE 30-52** Blast crisis of chronic myelocytic leukemia with myeloblasts in CSF.



■ **FIGURE 30-53** High-grade lymphoma, small noncleaved cell type, CSF, pleural fluid.

blood contamination. If no erythrocytes are present, even a low number of blasts (1–2%) can indicate CNS involvement.[35] Special studies such as cytochemistry stains, TdT, and surface markers by immunoperoxidase stains or flow cytometry can be helpful[21] (Chapters 23 and 40).

Any type of lymphoma can involve the CNS, but the high-grade lymphomas such as lymphoblastic, diffuse large B-cell, and Burkitt lymphoma are more often seen. Primary CNS lymphoma is seen more often in patients who are HIV-positive. The lymphomas in these patients are high grade and frequently correspond to Burkitt lymphoma or diffuse large B-cell lymphoma (Figure 30-53 ■).

The most common yeast organism seen in CSF is *cryptococcus* (Web Figure 30-7). When *cryptococcus* is suspected from the cytocentrifuge-prepared slide, the microbiology laboratory should perform an India ink preparation to confirm the presence of the characteristic large capsule of *cryptococcus.* If very few organisms are present, however, the India ink preparation can be negative because unconcentrated CSF is used. Cultures must be obtained to confirm the type of organism present.

CASE STUDY *(continued from page 590)*

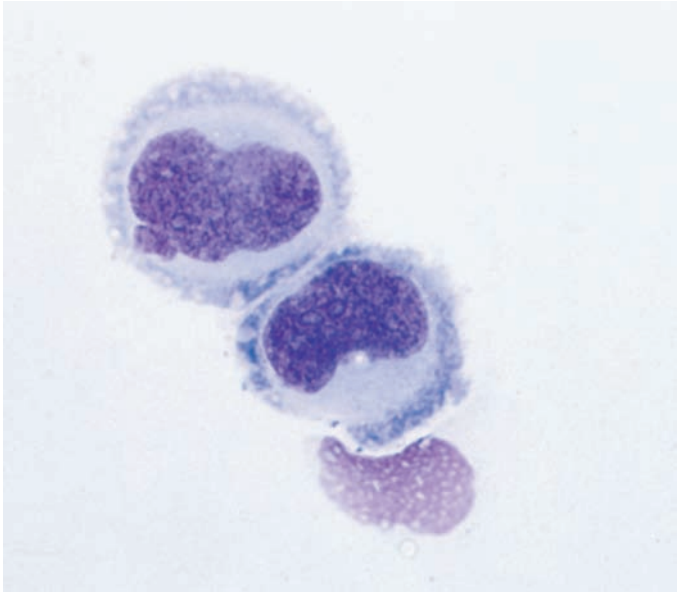
After an extensive work up, Carolyn is found to have a primary adenocarcinoma of the right upper lung lobe. A surgical resection is performed. Six months later, the patient has severe headaches. A spinal tap is performed, and a photomicrograph of the cells is as seen in Figure 30-54 ■.

7. What is the most appropriate interpretation of these cells?

Joint Fluid

The tissue cells present in joint fluids are synovial lining cells and have a similar appearance to mesothelial cells with a somewhat denser cytoplasm (Figure 30-55 ■). While they can resemble mesothelial cells, mesothelial cells only occur in the serous spaces and are not present in the synovial spaces. Synovial cells can become proliferative in a reactive setting similar to mesothelial cells. Reactive synovial cells also can be multinucleated and occur in clusters, and their nuclei can have small, regular nucleoli. Malignant cells can be seen in synovial fluid; however, this is extremely rare.[36]

The so-called rheumatoid arthritis (RA) cell seen in synovial fluid is a neutrophil containing granules of immune complexes.

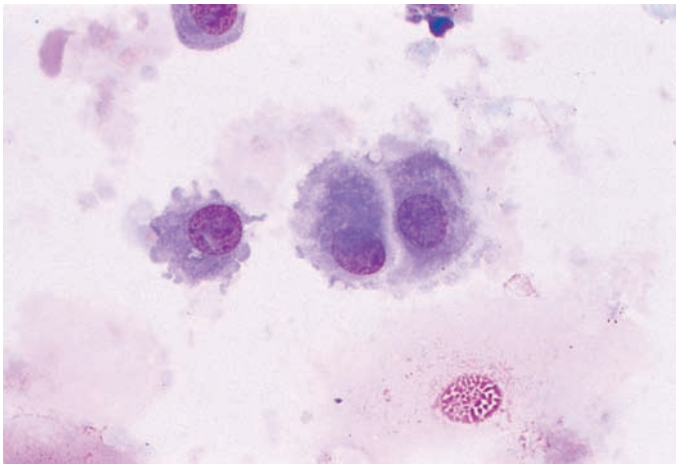


■ **FIGURE 30-54** Cerebral Spinal Fluid (CSF).

These cells are not specific for a diagnosis of RA. The Reiter's cell is a macrophage with vacuoles containing debris of phagocytosed neutrophils. The debris also can be unrecognizable blue material. These cells are also nonspecific and not diagnostic for Reiter's disease and are usually classified as neutrophages or monomacrophages.

Although crystals can be seen on occasion in other body fluids (most commonly cholesterol in serous fluids), they have far greater diagnostic importance in joint fluid. The three most common types of crystals present in joint fluid are monosodium urate crystals seen in gout, calcium pyrophosphate crystals seen in pseudogout, and cholesterol crystals present in different types of chronic arthritides such as RA.[37] Examination for crystals on a cytocentrifuge-prepared slide is superior to a standard wet preparation because the cytocentrifuge concentrates the specimen. Samples that are negative with wet preparation can actually show crystals on the concentrated cytocentrifuge slide. Using cytocentrifuge-prepared slides also decreases the biologic hazard when handling wet preparations. Cytocentrifuge slides prepared for crystal examination should not be stained. Uric acid crystals are very hygroscopic and dissolve easily if any water or moisture is present in either the fixative or the stain. If sufficient numbers of crystals are present, they can be seen with plain light microscopy. However, polarized light must be used to confirm birefringence.[38] If fewer crystals are present, polarization is necessary to see them initially. Every joint fluid sent to the hematology laboratory for cell counts should have a crystal examination.[39] Although Wright-stain techniques can result in the dissolution of the monosodium urate crystals, calcium pyrophosphate crystals are not moisture sensitive and can readily be observed in stained preparations (Table 30-9 ★).

Birefringence refers to a particular material's ability to refract light rays. It is determined by using a polarizing microscope. A fixed light filter (analyzer) is placed above the specimen and a rotating filter (polarizer) is placed below the specimen. Both filters allow light to pass in only one direction. When the polarizer is rotated 90 degrees to the analyzer, no light can pass, yielding a "dark field." If the specimen contains birefringent material, it changes the direction (refracts) of the light rays, allowing them to pass through the analyzer, and the birefringent material is seen as a bright particle or crystal.[38] A quartz compensator, often referred to as *1 red compensator filter*, is used to further identify a crystal by determining the velocity of the light rays passing through the crystal's grain (axis).



■ **FIGURE 30-55** Synovial cells in joint fluid.

Monosodium urate (MSU) crystals should be reported as intracellular and/or extracellular. MSU crystals are typically long, thin, and needlelike with pointed ends (Figure 30-56a ■). They can be seen singly or in bundles. MSU crystals are strongly birefringent and are brilliant with polarized light (Figure 30-56b). A quartz compensator must be used to determine positive or negative birefringence. MSU crystals are negatively birefringent and when aligned parallel to the axis of the compensator, show a yellow color; when turned perpendicular to the axis of the compensator, the color changes to blue [2,7,38] (Figure 30-56c).

Calcium pyrophosphate (CPP) crystals also can be seen intracellularly and/or extracellularly. CPP crystals are typically short, rectangular, and weakly birefringent, so they can be difficult to see with polarized light (Figures 30-57a and 30-57b ■). When aligned parallel to the axis of a quartz compensator, the CPP crystals are blue but change to yellow when they are perpendicular to the axis of the compensator [2,7,38](Figure 30-57c). A joint fluid occasionally has both MSU and CPP crystals; the presence of one does not exclude the other.

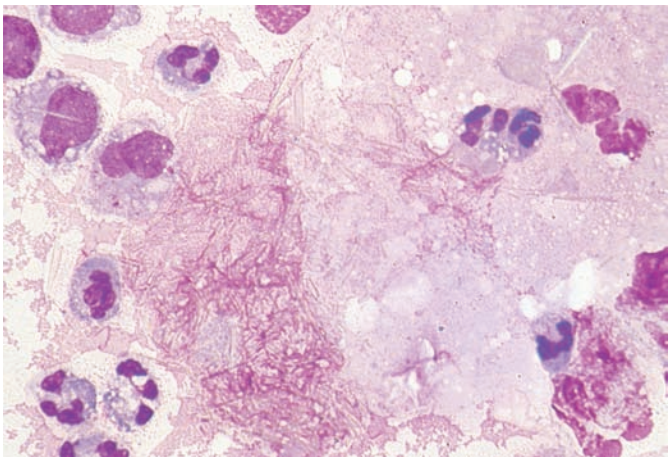
Cholesterol crystals have a characteristic notched-plate shape and are birefringent (Figure 30-58 ■). These crystals are present in chronically inflamed joints as are seen in RA.

Starch particles are distinguished from pathogenic crystals by a characteristic Maltese cross shape with polarized light (Figure 30-19).

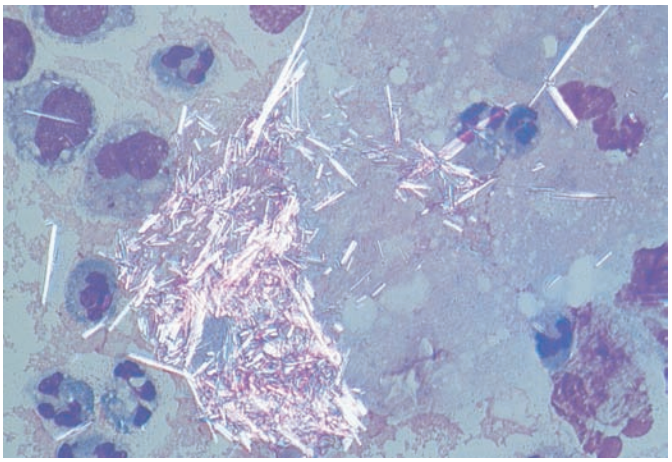
If a joint has been injected with steroids, the steroid particles can be seen intracellularly and extracellulary. Steroid particles do not have a crystal shape and are amorphous but birefringent (Figure 30-59 ■).

★ **TABLE 30-9** Morphologic Comparisons of Commonly Seen Birefringent Crystals and Particles

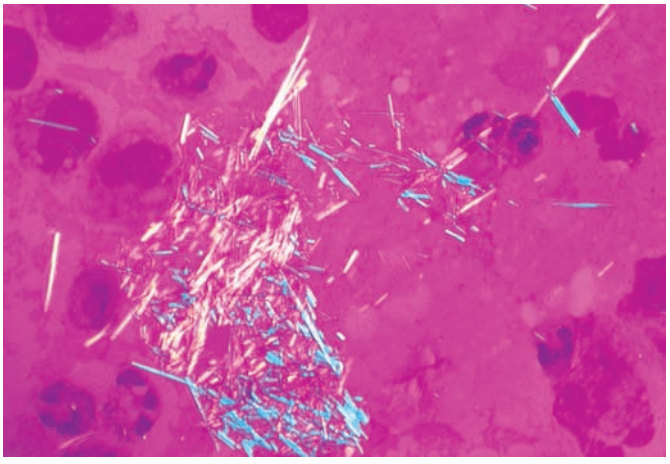
Crystal	Birefringence	Color Parallel to Quartz Compensator	Morphology
Monosodium urate	Strong	Yellow	Long, thin, needlelike, intra- and extracellular
Calcium pyrophosphate	Weak	Blue	Short, rectangular, intra- and extracellular
Cholesterol	Strong	Variable	Large platelike, notched, extracellular
Steroids	Strong	Variable	Amorphous, intra- and extracellular
Talc particles	Strong	Yellow and blue	Maltese cross shape, extracellular



a

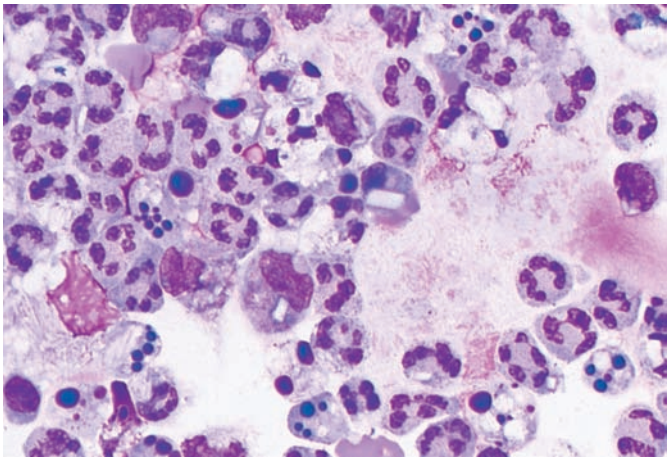


b

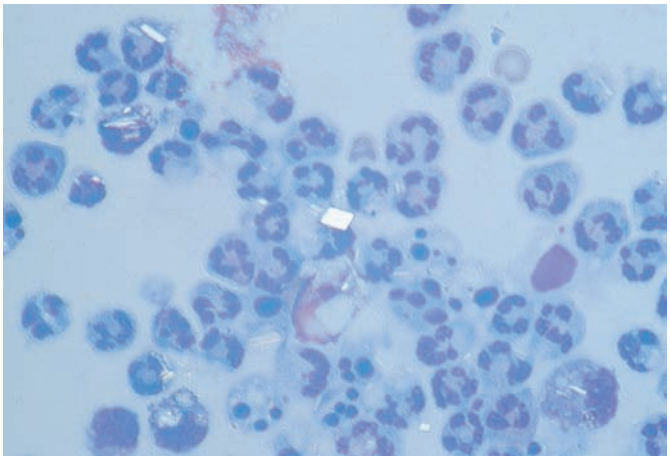


c

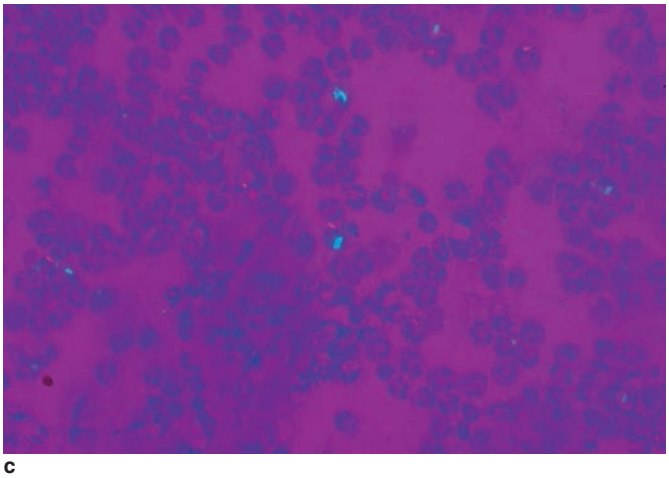
FIGURE 30-56 (a) MSU crystal with plain light, (b) polarized light, (c) quartz compensator with yellow crystal parallel to quartz line and blue crystal perpendicular to quartz line.



a

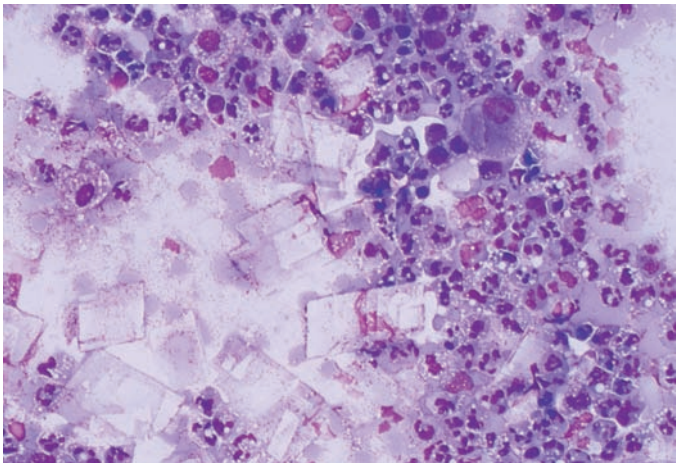


b

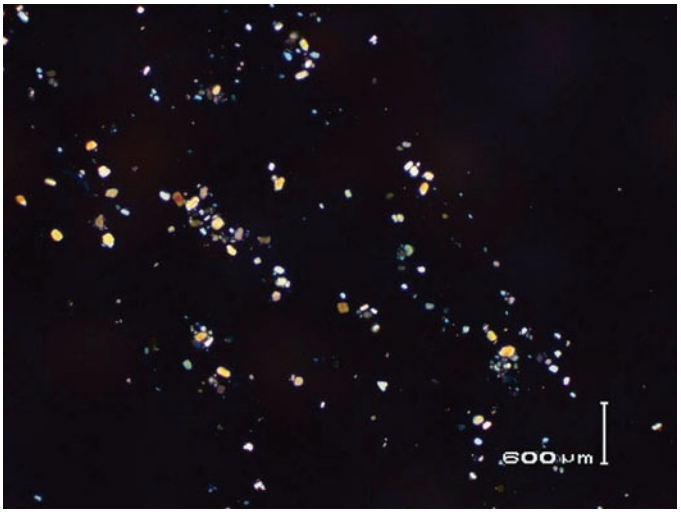


c

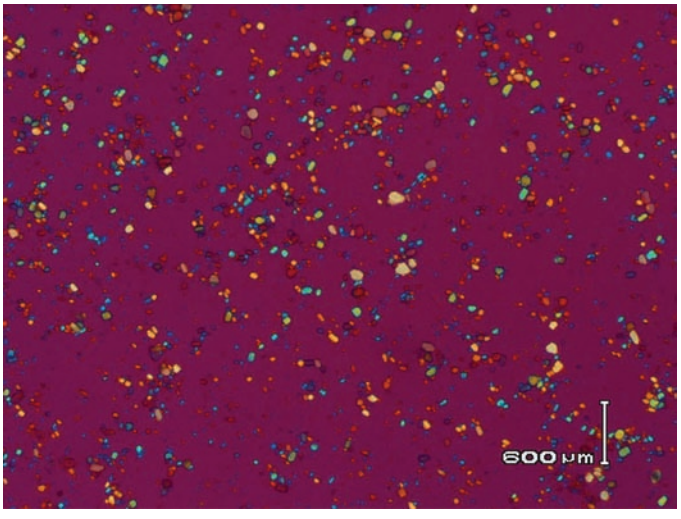
FIGURE 30-57 CPP crystal with (a) plain light, (b) polarized light, (c) quartz compensator with yellow crystal perpendicular to quartz line and blue crystal parallel to quartz line.



■ **FIGURE 30-58** Cholesterol crystals in joint fluid.

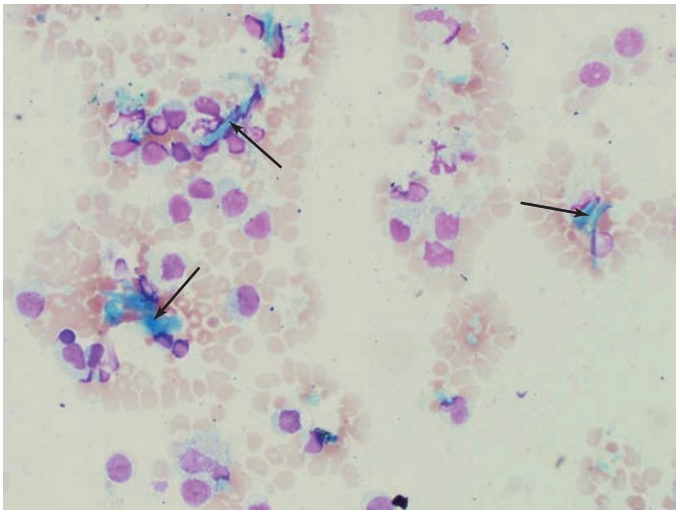


a



b

■ **FIGURE 30-59** Steroid particles in joint fluid: **(a)** polarized light, **(b)** quartz compensator.

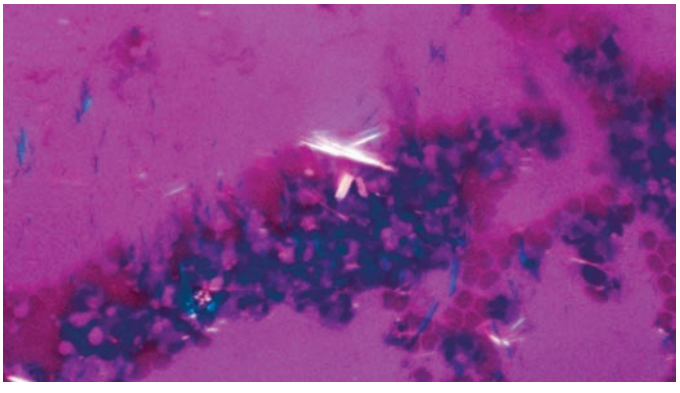


■ **FIGURE 30-60** Polyethylene pieces (arrow) in joint fluid.

A microscopic finding that can be important in joint fluids is the presence of polyethylene fragments. These fragments, essentially pieces of plastic, stain bright blue with most Wright stains and appear as artifact or a contaminant, which they can be because plastic devices are used to obtain and process most fluids (syringes, cytocentrifuge chambers, etc.). However, plastic is also a component in artificial joint replacements, and increased numbers of polyethylene pieces seen in the fluid in these cases can indicate failure of the replacement joint (Figure 30-60 ■).

☑ CHECKPOINT 30-5

A 57-year-old man has an acutely swollen, painful, reddened joint in his left great toe. Joint fluid is aspirated, and a photomicrograph is taken (Figure 30-61 ■). This picture is taken with polarized light using a quartz compensator. What is the most appropriate interpretation of this finding?



■ **FIGURE 30-61** Synovial fluid.

ANALYSIS OF OTHER FLUIDS

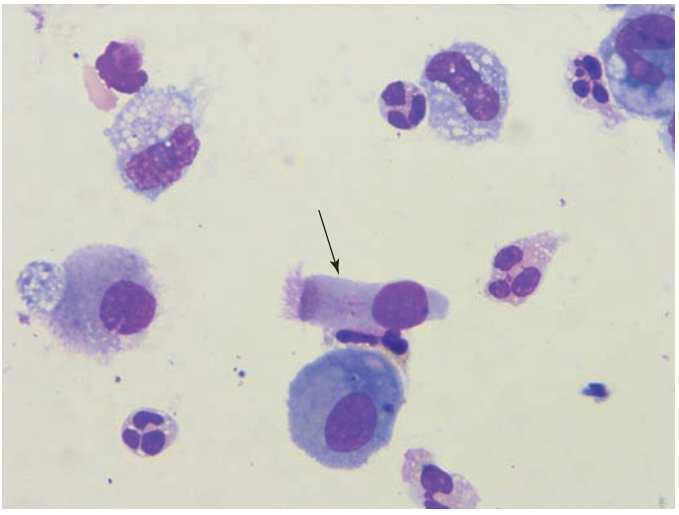
The hematology laboratory usually is responsible for performing analysis on other fluids including BAL fluid for determining conditions in the lower respiratory tract, amniotic fluid lamellar body counts to determine fetal lung maturity, and semen to assess the number and morphology of sperm as well as other characteristics of the fluid.

BAL Fluid

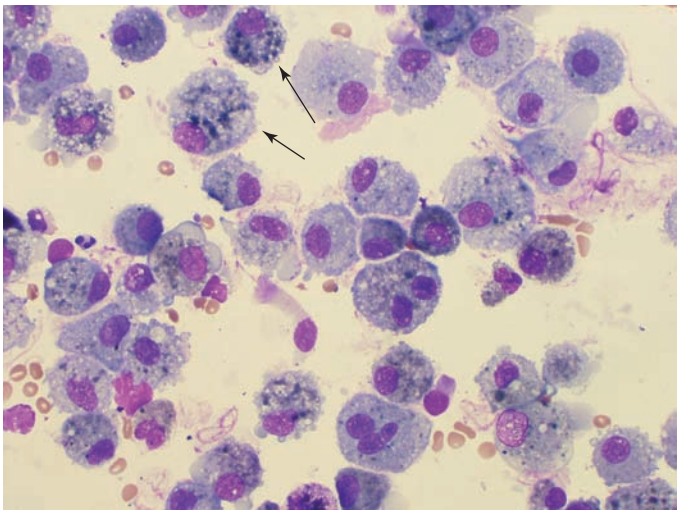
Tissue cells seen in BAL samples are bronchial lining cells. They are considered a contaminant in lavage samples because they indicate sampling from the bronchial tree rather than the lower respiratory tract.[1] Bronchial lining cells are unique in that they have a row of cilia at one end (Figure 30-62 ■). The cilia can become detached because of normal physiological processes and appear as unattached tufts called *ciliocytophthoria*. Ball-like clusters of bronchial lining cells are referred to as *creola bodies* and can be confused with malignant cells. The presence of the cilia should help correctly identify these cells as bronchial lining cells. BAL samples from smokers show increased monomacrophages, many with irregular, black inclusions. This is often called "smokers lung," and the inclusions are carbon particles. They should not be mistaken for hemosiderin or a microorganism (Figure 30-63 ■). Epithelial cells can be seen but also are considered a contaminant of the procedure primarily from the upper airway. The laboratory professional typically includes these contaminant cells in an "other" category of the differential.

Amniotic Fluid Lamellar Body Counts

Lamellar body counts (LBCs) in amniotic fluid are an assessment of fetal lung maturity (FLM) and the associated risk of developing respiratory distress syndrome (RDS) in the premature newborn. RDS is caused by insufficient surfactant in the newborn's lungs. Lamellar bodies are densely packed layers of phospholipid secreted by the type II alveolar cells and are the storage packages for surfactant in amniotic fluid. The number of lamellar bodies present in the amniotic fluid is proportional to the amount of surfactant available. Lamellar bodies are similar in size and density to platelets and can be counted in the platelet channel of most hematology analyzers.[40] A meta-analysis of studies showed that the LBCs performed equally as well, and in some cases, better than the lecithin-to-sphingomyelin (L:S) ratio in predicting RDS.[41]



■ **FIGURE 30-62** Ciliated bronchial lining cell (arrow), neutrophils, and monomacrophages in bronchoalveolar lavage sample.



■ **FIGURE 30-63** Bronchoalveolar lavage: monomacrophages with carbon inclusions (arrows) in smoker's lung.

Lamellar bodies can be counted by both impedance and optical platelet-counting methodologies. However, it is critical that the laboratory establish a maturity cutoff for the methodology used.[42] As with previous FLM methods, two different cutoffs need to be determined:

- One cutoff to indicate maturity when the LBC is higher than the cutoff limit
- A second cutoff to indicate immaturity when the LBC is less than the cutoff limit

LBCs that fall between the two cutoffs are considered indeterminate.

According to CLSI document C58-A,[43] three approaches to establishing the cutoffs are acceptable. The first is to compare LBCs to newborn outcomes. This approach requires access to newborn records and cooperation with neonatologists in accurately defining RDS. Because of the low incidence of RDS, this method can take considerable time.

A second approach to establishing the cutoffs is to compare the LBC to an existing FLM method such as the L:S ratio. The lamellar body cutoff that consistently classifies samples as mature when compared with the existing method can appropriately be used for the mature cutoff. This approach can require access to newborn medical records to resolve any discrepancies between the two methods.

The third approach is split sample analysis with a laboratory that is already performing and reporting a validated lamellar body count. Various published cutoffs list a mature LBC cutoff of 40,000–50,000/mcL and an immature cutoff of 15,000–19,000/mcL. Values between these cutoffs are reported as indeterminate. It should be noted that the majority of published references used an impedance platelet-counting methodology, and optical counts can vary significantly.

Known interfering substances are meconium and blood. Samples contaminated with meconium are not acceptable for LBC analysis because meconium contamination causes a falsely elevated LBC. Blood, in sufficient quantity, causes a paradoxical biphasic interference resulting in an initial increase in LBCs (presumably from the introduction of peripheral platelets) followed by a decrease in LBCs most likely because of the lamellar bodies trapped in fibrin strands. Samples contaminated with blood can be tested for LBCs if the RBC count is <31,000/mcL.[44] An aliquot of the well-mixed amniotic fluid sample should be placed in a clear tube, if necessary, to observe the sample for blood or meconium contamination. LBCs in amniotic fluid are stable for 33 days if refrigerated (4°C). The sample should not be frozen or centrifuged because either of these actions falsely decrease the LBC.[43,44]

To perform the LBC, the sample is mixed by inversion for a minimum of 10 times and analyzed on an automated hematology instrument that provides a histogram or scattergram display of the platelet channel result. The histogram or scattergram display should be examined for acceptability of results as with all samples analyzed on an automated hematology instrument.

An example of an amniotic fluid LBC with acceptable platelet impedance histogram is shown in Figure 30-64 ■. An example of an unacceptable platelet impedance histogram indicating probable interference with the accuracy of the result is seen in Figure 30-65 ■. In these instances, the LBC cannot be performed because of interfering substances. The platelet channel result, if acceptable, is reported as the LBC.

Vaginal pool samples can be tested for lamellar bodies but are not the recommended sample. Vaginal pool samples can be contaminated with blood, mucus, bacteria, or urine, which can have unpredictable effects on the LBC. If it is necessary to test a vaginal pool sample, the specimen type/source should be clearly indicated on the report.[43] Commercial QC and proficiency testing are available for LBCs.

Semen Analysis

Semen analysis is an important test in assessing male fertility. Semen is the fluid discharged during ejaculation consisting of secretions from glands and containing the male mature gamete **sperm,** also called spermatozoa. The routine laboratory should perform a basic battery of tests on the semen that includes volume measurement, appearance, liquefaction time, viscosity, pH, sperm motility, sperm count and morphology evaluation, and, when indicated, sperm viability. The more esoteric tests used in diagnosing and treating infertility should be performed in an andrology laboratory and are not covered in this section.

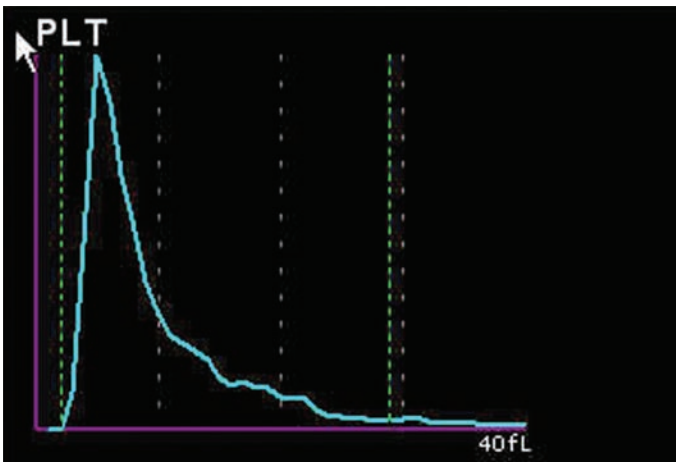
Historically, methods and reference intervals for semen analysis have varied widely. To address this problem, the World Health Organization (WHO) published a manual of standard methods in 1989, *The WHO Laboratory Manual for the Examination and Processing of Human Semen*. The manual is now in its fifth edition and is considered a global standard for semen analysis. Methods discussed here are based on the *WHO Laboratory Manual*, fifth edition.[45]

Physical and Chemical Properties

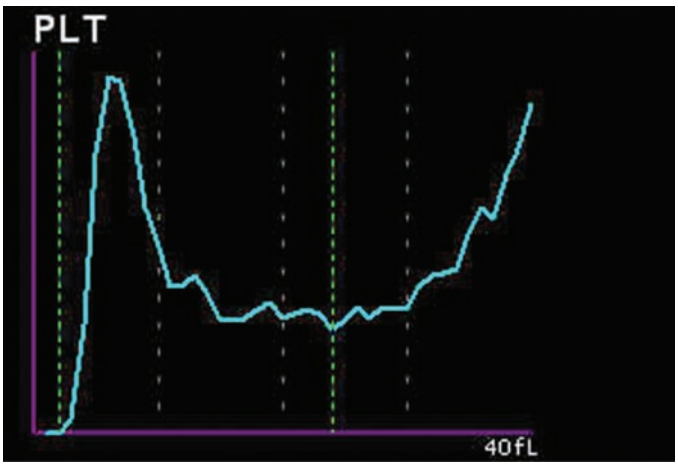
Samples for routine semen analysis should be collected after a period of abstinence of a minimum of 2 days to a maximum of 7 days. The recommended method of collection is by masturbation. Samples collected in ordinary condoms are not acceptable. Condoms acceptable for semen analysis are commercially available. Samples should be collected into a clean, wide-mouth container made of glass or plastic. Anecdotal information suggests that some plastics can have a toxic effect on semen, resulting in a decreased motility assessment. Plastic containers provided by the laboratory should be tested to verify nontoxicity by performing split sample analysis (glass and plastic) for motility on several high sperm count samples. Best practice should require all variables—volume of sample received in the laboratory, method of collection, type of container, and days of abstinence—to be included in the reported results.

Volume and Appearance

The lower reference interval for semen volume is 1.5 mL. The appearance of the semen sample should be reported. Normal semen has a homogenous grey-opalescent appearance and a report of "normal" is sufficient. A sample that is "abnormal" in color can be reported as such but should be described. A red- or brown-tinged sample usually indicates the presence of blood. Jaundice, urine contamination, and some vitamins and drugs can result in a yellow color.



■ **FIGURE 30-64** Impedance platelet (PLT) channel histogram showing acceptable analysis.



■ **FIGURE 30-65** Impedance platelet (PLT) channel histogram showing unacceptable analysis with interference on the right slope of the histogram.

Liquefaction

Normal semen is a congealed substance when ejaculated and begins to liquefy within minutes. The liquefaction allows the sperm to become motile. Total liquefaction should occur in 15 to 60 minutes and the sample should be thin, runny, and homogeneous. Gelatinous particles can be seen in normal liquefied semen sample.

Samples that do not liquefy impede the accurate measurement of sperm motility and count. Suggested methods to liquefy abnormal semen include treatment with a proteolytic enzyme such as chymotrypsin or bromelain.

Viscosity

Viscosity should be measured after the sample has liquefied. Semen drawn into a disposable transfer pipet should slowly dispense in discrete drops if viscosity is normal. Semen with abnormal viscosity forms a thread at least 2 cm in length when dispensed from the pipet. Viscosity is usually reported as normal or increased.

pH

Semen pH should be measured with a pH paper or indicator strip with a range of 6.0–10.0, ideally at 30 minutes but acceptable up to 1 hour postejaculation. Whether the laboratory uses the 30-minute or 1-hour limit, all samples should be tested within the same time constraint. Normal semen is alkaline and has a pH 7.2 or higher.

Microscopic Analysis

After the physical and chemical properties of the semen have been analyzed and recorded, slides are made to examine the fluid microscopically. In addition to the sperm count, the sperm are examined for motility, viability, and morphology.

Motility

Motility of the sperm should be assessed after liquefaction ideally at 30 minutes, but acceptably at 1 hour postejaculation. Again, all samples should be tested at a consistent time after ejaculation. The semen is mixed well by inversion and at a standard volume such as 10 mcL or one drop from a standard transfer pipet placed onto a clean microscope slide. A standard coverslip (e.g., 22 × 22 mm for a 10-mcL volume) is added and the sample allowed to spread on the slide. The goal of the standard sample volume and standard coverslip is to achieve an approximate depth of 20 mcM (μm) between the slide and coverslip. A depth of <20 mcM hinders the movement of the spermatozoa. A method for calculating the depth based on sample volume and coverslip size can be found in the *WHO Laboratory Manual*.[45]

Motility should be determined using 400× magnification. Two hundred sperm are counted in random fields and classified as:

- Progressive (moves forward latterly or in a straight line)
- Nonprogressive (movement but no progression in any particular direction)
- Nonmotile (no movement at all)

The process is repeated on another 200 sperm, and the percentage for each category is averaged. The average percentage for each of the three motility grades is reported. The lower reference limit for total motility (progressive and nonprogressive) is 40%. The lower reference limit for progressive motility is 32%.

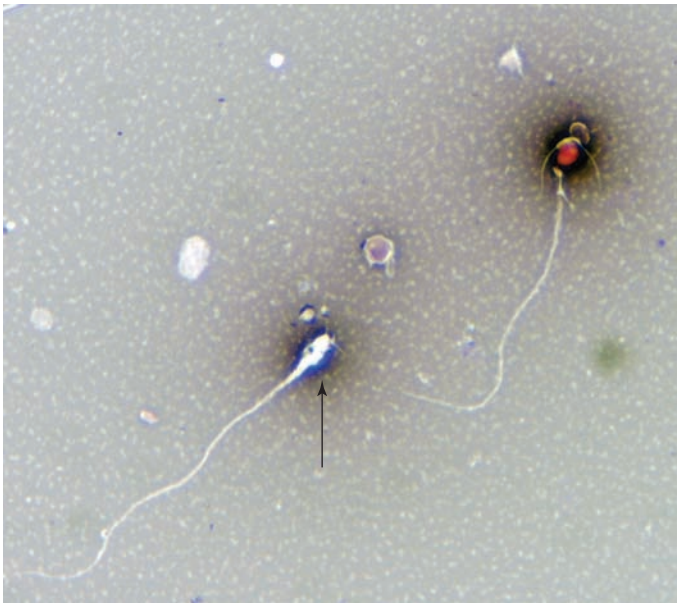
When examining the slide for sperm motility, abnormal agglutination of the sperm should be noted if present. *Agglutination* refers to motile sperm sticking to each other, head to head, tail to tail, or in a mixed array. Agglutinated sperm are often robustly motile, appearing as a shaking clump of sperm with little true motility. Motile spermatozoa that are agglutinated in noticeable clumps should be reported.

Vitality

Sperm vitality (also known as *viability*) is a measurement of the percentage of live spermatozoa. A nigrosin-eosin stain is used to identify nonvital sperm, which have damaged cell membranes that allow the eosin stain to enter and stain the sperm head pink. The intact cell membranes of vital sperm prevent the dye from entering leaving the sperm head unstained. The nigrosin provides a dark background, so the vital sperm appear white (Figure 30-66 ■). The vitality stain should be performed on all samples that have less than normal progressive motility. WHO suggests performing the stain on samples with <40% progressive motility. After staining the specimen and making a smear, 100 sperm are counted for vitality at 400×. The average percentage of vital sperm (unstained forms) is reported; the lower reference limit is 58%.

Sperm Count

Sperm counts should be performed only after the semen sample has liquefied. Traditionally, sperm counts have been performed using the hemacytometer, and the usual counting rules can be applied with the caveat that the only information that matters is where the sperm head



■ **FIGURE 30-66** Nigrosin-eosin stain for sperm vitality showing an unstained vital (live) sperm (arrow) and a pink-stained nonvital sperm.

★ **TABLE 30-10** Criteria for Normal Sperm Morphology

Head	Mid-piece	Tail
Smooth and generally oval	Slender	Uniform in diameter
Length 4.0–5.0 mcM (μM)	Approximately same length as the head	Thinner than the mid-piece
Width 2.5–3.5 mcM	Axially attached to the head	Approximately 10 times the length of the head (45 mcM)
Length to width ratio 1.3–1.8	Residual cytoplasm less than one-third the size of the head	May be looped back on itself but no sharp angles.
Acrosome occupies 40–70%		
Acrosome contains no large vacuoles and not >2 small vacuoles occupying <20% of the head		
Postacrosome occupies 30–60% and contains no vacuoles		

lies in regard to which sperm to include in the count. The position of the tail is irrelevant. A typical dilution used for counting sperm on a hemacytometer is 1:20. Dilutions might need to be adjusted for very high or very low counts with a goal of counting at least 200 sperm in the chamber to reduce sampling errors. Counts should be done in duplicate as well as in a duplicate dilution. The standard formula for determining hemacytometer counts is used, but the standard formula calculates the result per microliter, whereas sperm counts are reported per milliliter. The calculated result from the hemacytometer must be multiplied by 1000 to convert the microliter result to a per milliliter result.

Newer dedicated and disposable sperm-counting chambers such as the Microcell™ and Cell-Vu™ are commercially available that simplify and standardize the diluting and counting process.

Regardless of the method used to perform the count, the sperm need to be immobilized before counting. Methods for immobilizing sperm include heating an aliquot at 56°C for 5 minutes or adding a drop of formalin to a small amount of saline or other diluting fluid used to make the counting dilution. An important step in performing any type of manual sperm count is to ensure that the semen sample is well mixed before a portion is removed for dilution or counting. Only intact sperm—those with a head and tail—are included in the count. The appearance of numerous unattached tails should be noted. Automated methods for performing semen analysis are recently available (SQA-V^{TM} and QwikCheck^{TM}); they are capable of performing sperm motility, count, and morphology. Although most laboratories identify the number of sperm per mL as the "sperm count," WHO considers this the "sperm concentration." According to WHO, total sperm number is the total number of sperm in the entire ejaculate and is calculated by multiplying the sperm concentration (number per milliliter) by the semen volume. The lower reference limit for sperm count is 15×10^6/mL.

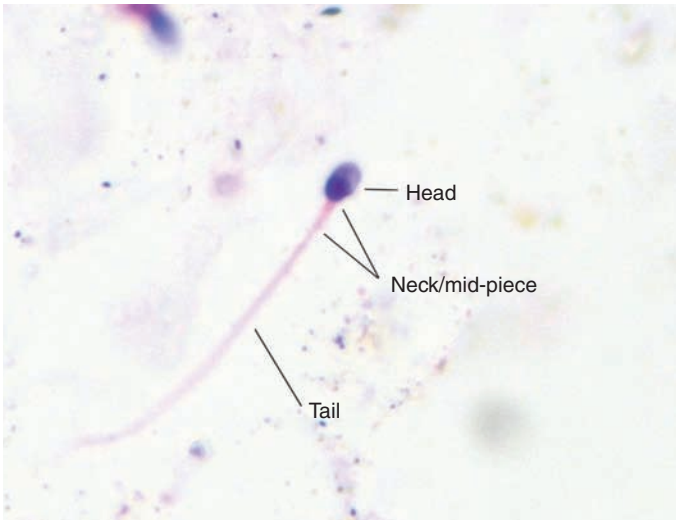
Morphology

Slides for morphologic examination of sperm should be made after liquefying the sample. A drop of well-mixed semen should be placed on a slide and a second slide used to make a pull-type smear. Slides should be made in duplicate. If the sperm count is very low, a portion of the sample can be concentrated by centrifugation before slide preparation. The slides should be allowed to dry before staining. The preferred stain is the Papanicolaou stain although others (Shorr, Diff-Quik) are acceptable.

When visualized by light microscopy, with the Papanicolaou stain, spermatozoa consist of a head, neck (mid-piece), and tail (principal piece). The head has a light-staining area at the top called the *acrosome* and a darker staining area at the bottom called the *postacrosomal area*. The head is attached to the tail by the neck or mid-piece. With the Papanicolaou stain, the sperm head is stained pale blue in the acrosomal region and darker blue in the postacrosomal region. The mid-piece and the tail are stained a pinkish color. Excess residual cytoplasm, usually located behind the head and around the mid-piece, is stained pink.

Various schemes have been used for assessing sperm morphology, described as "liberal" (MacLeod and GOLD) and "strict" (Tygerberg, Kruger). WHO defines *morphology* according to strict criteria. Refer to Table 30-10 ★ for criteria for a morphologically normal sperm.

Both the head and tail must be normal for a sperm to be considered normal (Figure 30-67 ■). Abnormalities of the sperm head include size and shape defects as well as excess vacuoles (Web Figure 30-19). Abnormalities of the mid-piece include defects in thickness, asymmetrical attachment to the head, and sharp kinks or bends (Web Figure 30-20). Tail abnormalities include defects in length, width, and shape (sharp bends, coils) (Web Figure 30-21). Questionable or



■ **FIGURE 30-67** Normal sperm, Papanicolaou stain.

borderline forms should be considered abnormal. Any apparent predominant abnormality—head, neck, or tail—should be noted. The lower reference limit for normal forms is 4%.

Other cellular material that can be seen in stained semen slides include WBCs, spermatocytes, and spermatids. Appearance of more than a few WBCs in each microscopic field should be noted. Spermatocytes and spermatids are precursors of the mature sperm and often look like nucleated RBCs on stained preps (Web Figure 30-22). If more than a rare spermatid is seen, it should be noted on the report.

Summary

The fluids discussed in this chapter are those commonly seen in the routine clinical laboratory. Serous fluids include pleural, peritoneal, and pericardial fluids. Other fluids examined are synovial fluids, CSF, amniotic fluid, semen, and BAL. In pathologic conditions, the amount of serous and synovial fluid (effusion) can increase. An effusion can be a transudate or an exudate. Traditional criteria for differentiating transudates from exudates include specific gravity measurements and leukocyte counts. The current criterion for pleural effusions, known as *Light's criteria*, is to use serum and fluid measurements for protein and LDH. Chylous effusions are milky and result from leakage of lymphatic vessels. Common cell types seen in these fluids include WBCs, tissue cells, and malignant cells. Examination of cellular morphology in body fluids, a critically important procedure for hematology laboratories, includes not only a differential leukocyte count but also a possible demonstration of diagnostic findings, such as micro-organisms and malignant cells. The cytocentrifuge-prepared Wright-stained slide yields excellent morphology of cells and can significantly aid in making a timely diagnosis of patients with effusions of unknown etiology. The hematology laboratory must take an active role in correlating morphologic findings with any additional studies performed, such as special stains, cytology, and cultures.

Review Questions

Level I

1. The type(s) of body fluids other than peripheral blood frequently sent to the hematology laboratory can include which of the following? (Objective 1)
   A. cerebrospinal fluid
   B. pleural fluid
   C. pericardial fluid
   D. all of the above
2. The pleura, pericardium, and peritoneum are composed of what type of cell? (Objective 4)
   A. white blood cell
   B. epithelial cell
   C. mesothelial cell
   D. squamous cell
3. Anatomically, where are the visceral pleura located? (Objective 1)
   A. innermost aspect of the abdominal wall
   B. outermost portion of the heart
   C. innermost aspect of the chest wall
   D. outermost portion of the lung
4. Which of the following cell types seen in cerebrospinal fluid can be considered normal, not a sign of pathologic disease? (Objective 4)
   A. arachnoid cells
   B. choroid plexus cells
   C. ependymal cells
   D. all of the above
5. An LBC between 19,000–40,000 is: (Objective 10)
   A. indeterminant
   B. an indication of fetal lung maturity
   C. an indication of fetal lung immaturity
   D. the high reference level
6. Which of the following can be seen as artifact(s) on a cytocentrifuged, Wright-stained slide? (Objective 5)
   A. hypersegmentation of neutrophils
   B. stain precipitate
   C. cytoplasmic projections of lymphocytes
   D. all of the above

7. The sperm concentration is reported as: (Objective 9)
   A. number per microliter
   B. number per liter
   C. number per deciliter
   D. number per milliliter
8. A CSF that appears orange should be described as: (Objective 2)
   A. xanthrochromic
   B. hazy
   C. bloody
   D. an old and unacceptable sample
9. Malignant tissue cells have which of the following morphologic feature characteristic(s)? (Objective 7)
   A. irregular nuclear membrane
   B. evenly distributed chromatin
   C. prominent, irregular nucleoli
   D. a and c only
10. True pathogenicity versus in vitro contamination of the body fluid specimen can be demonstrated by: (Objective 6)
    A. finding the organisms intracellularly
    B. staining the specimen purple with Wright stain
    C. staining the specimen red with Gram's stain
    D. finding the organisms in cytocentrifuged specimens

Level II

1. A specimen labeled "ascites" is sent to the laboratory. What type of procedure was used to obtain this fluid? (Objective 1)
   A. thoracentesis
   B. lumbar puncture
   C. pericardial aspiration
   D. paracentesis
2. Which of the following cell types is responsible for the production of cerebrospinal fluid? (Objective 2)
   A. choroid plexus
   B. arachnoid
   C. neutrophil
   D. mesothelial
3. A 25-year-old woman develops a left-sided pleural effusion while recovering from bacterial pneumonia. A thoracentesis is performed, and the following laboratory results are reported:

	Serum	Fluid
Protein	6.5 g/dL	5 g/dL
LD	75 U/L	60 U/L

   These results would be best interpreted as which of the following? (Objective 3)
   A. chylous
   B. exudate
   C. pseudochylous
   D. transudate
4. A 56-year-old woman has a 3-week history of abdominal pain. A peritoneal effusion is found. After paracentesis, a sample is sent to the laboratory. Examination of the cytocentrifuged, Wright-stained slide shows clusters of large cells that have abundant cytoplasm, smooth nuclear membranes, evenly distributed chromatin, and no nucleoli. An occasional multinucleated cell is found with the same features. These cells most likely represent: (Objective 6)
   A. reactive mesothelial cells
   B. adenocarcinoma cells
   C. small-cell carcinoma
   D. ependymal cells
5. A 60-year-old man who has smoked cigarettes since he was 16 years old has left-sided chest pain. A thoracentesis sample is sent to the laboratory. The cytocentrifuged, Wright-stained slide examination reveals clusters of large cells that have irregular, jagged nuclear membranes, prominent and irregular nucleoli, and unevenly distributed chromatin. These cells most likely represent: (Objective 6)
   A. reactive mesothelial cells
   B. adenocarcinoma cells
   C. large-cell lymphoma
   D. reactive histiocytes
6. Referring to the case in question 5, the protein, LD, and other studies would most likely reveal that this is a(n): (Objective 3)
   A. exudate
   B. chylous fluid
   C. transudate
   D. pseudochylous fluid

7. A 65-year-old woman has a painful, swollen elbow. A joint aspiration is performed, and a sample is sent to the laboratory. A cytocentrifuged slide is examined with polarized light and a quartz compensator, revealing birefringent crystals intracellular and extracellular; they are needlelike in appearance and are yellow when oriented parallel to the quartz compensator. These crystals would be best identified as which of the following? (Objective 8)

   A. calcium pyrophosphate
   B. cholesterol
   C. monosodium urate
   D. steroids

8. Referring to the case in question 7, which of the following is the most likely diagnosis? (Objective 8)

   A. gout arthritis
   B. pseudogout arthritis
   C. rheumatoid arthritis
   D. systemic lupus erythematosus

9. A 55-year-old man is brought to the emergency room in a comatose state. He was found at home by his son and appears to have fallen from a ladder. A spinal tap is performed and reveals the following:

Color	Red
Appearance	Bloody
RBC	100×10^9/L
WBC	0.10×10^9/L
Differential WBC	80% segmented neutrophils
	15% lymphocytes
	5% monocytes

   Which of the following would be the most specific finding in this patient for a true CNS hemorrhage versus traumatic spinal tap? (Objectives 7, 10)

   A. crenated red blood cells
   B. xanthochromia of the supernatant
   C. erythrophagocytosis by histiocytes
   D. WBC count of 0.1×10^9/L

10. Referring to the patient in question 9, what is the significance of the WBC count of 0.1×10^9/L? (Objectives 2, 4, 10)

    A. It is diagnostic for bacterial meningitis.
    B. It is diagnostic for early viral meningitis.
    C. It is expected for the amount of hemorrhage.
    D. It is diagnostic for fungal meningitis.

11. A 45-year-old man has pleural effusions on the right and left side. A right-sided thoracentesis is performed and a sample is sent to the laboratory. The fluid-serum protein ratio is 0.3, the fluid-to-serum LD ratio is 0.4, and the total nucleated cell count is low. Which of the following best describes this fluid? (Objective 3)

    A. chylous
    B. exudate
    C. pseudochylous
    D. transudate

Companion Resources

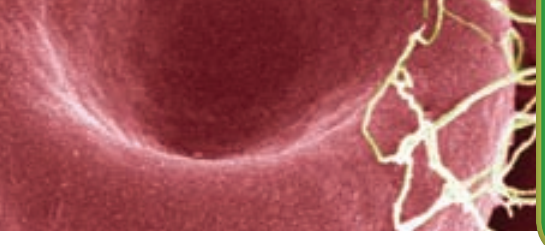
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Galagan KA, Blomberg D, Cornbleet PJ et al. *Color Atlas of Body Fluids*. Northfield, IL: College of American Pathologists; 2006.
2. Kieldsberg C, Knight J. *Body Fluids*, 3rd ed. Chicago: ASCP Press; 1993.
3. Light RW. Pleural effusion. *N Engl J Med*. 2002;346:1971–76.
4. McGibbon A, Chen GI, Peltekian KM et al. An evidence-based manual for abdominal paracentesis. *Dig Dis Sci*. 2007:52:3307–315.
5. Karcher DS, McPherson RA. Cerebrospinal, synovial, serous body fluids and alternative specimens. In: McPerson RA, Pincus MR, eds. *McPherson: Henry's Clinical Diagnosis and Management by Laboratory Methods*, 20th ed. Philadelphia: W. B. Saunders; 2011.
6. Morgenstern LB, Luna-Gonzales H, Huber JC et al. Worst headache and subarachnoid hemorrhage: prospective, modern computed tomography and spinal fluid analysis. *Ann Emerg Med*. 1998;32:297–304.
7. Arora S, Swadron S. Describing cerebrospinal fluid red blood cell counts in patients with subarachnoid hemorrhage. *West J Emerg Med*. 2007;8:102.
8. Heasley DC, Mohamed MA, Yousem DM. Clearing of red blood cells in lumbar puncture does not rule out ruptured aneurysm in patients with suspected subarachnoid hemorrhage but negative head CT findings. *Am J Neuroradiol*. 2005;26:820–24.
9. Julia-Sanchis ML. Rapid differential diagnosis between subarachnoid hemorrhage and traumatic lumbar puncture by D-dimer assay. *Clin Chem*. 2007;53:993.
10. Travis WD, Colby TV, Koss MN et al. Handling and analysis of bronchoalveolar lavage specimens with approach to patterns of lung injury. *ARP Atlases*. 2007;1:17–47.
11. Heron M, Grutters JC, ten Dam-MolenKamp KM et al. Bronchoalveolar lavage cell pattern from healthy human lung. *Clin Exp Immunol*. 2011;167:523–31.
12. Walker HK, Hall WD, Hurst JW. *Clinical Methods*, 3rd ed. Boston: Butterworths; 1990.

13. Sheaff MT, Singh N. *Cytopathology: An Introduction*. London: Springer-Verlag; 2013.
14. Perry JJ, Sivilotti MLA, Stiell IG et al. Should spectrophotometry be used to identify xanthochromia in the cerebrospinal fluid of alert patients suspected of having subarrachnoid hemorrhage? *Stroke*. 2006;37:2467–472.
15. Horn KD, Penchansky L. Chylous pleural effusions simulating leukemic infiltrate associated with thoracoabdominal disease and surgery in infants. *Am J Clin Path*. 1999;111:99–104.
16. Mundt LM, Shanahan K. *Graff's Textbook of Routine Urinalysis and Body Fluids*. Philadelphia: Lippincott Williams and Wilkins; 2011.
17. Steele R. Leukocyte survival in cerebral spinal fluid. *J Clin Microbiol*. 1986:23:965–66.
18. Geborek P, Truedsson L, Sandburg M et al. Hyaluronidase treatment of synovial fluid affects the recovery of mononuclear cells and their in vitro immunoglobulin synthesis. *Clin Exp Rheumatol*. 1987;5:255–57.
19. Galagan KA. Q&A. *CAP Today*. June 2012
20. Galagan KA. Q&A. *CAP Today*. January 2012.
21. Danise P, Maconi M, Rovetti A et al. Cell counting of body fluids: comparison between three automated haematology analysers and the manual microscope method. *Int J Lab Hematol*. 2013;35:608–13.
22. Williams JE, Walters J, Zajac K. Evaluation of the body fluid application on the Sysmex XE-5000 Hematology Analyzer. *Lab Med*. 2011;42(7):395–401.
23. Nakazawa N, Wada N, Tanaka Y et al. Evaluation of assay performance of the body fluid mode on the Automated Hematology Analyzer XN-2000. *Sysmex J Int*. 2012;22:8–18.
24. Shmerling RH, Delbanco TL, Tosteson A et al. Synovial fluid tests: what should be ordered? *JAMA*. 1990;264:1009–14.
25. Freemont AJ, Denton J, Chuck A et al. Diagnostic value of synovial fluid microscopy: a reassessment and rationalisation. *Ann Rheum Dis*. 1991;50:101–7.
26. Meyer KC, Raghu G, Baughman RP, et al. An official American Thoracic Society Clinical Practice Guideline: the clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease. *Am J Respir Crit Care Med*. 2012;185(9):1004–14.
27. Mitchell MA, Horneffer MD, Standiford TJ. Multiple myeloma complicated by restrictive cardiomyopathy and cardiac tamponade. *Chest*. 1993;103:946–47.
28. Nador RG, Cesarman E, Chadburn A et al. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood*. 1996;88:645–56.
29. Knowles D, Chadburn A. Lymphadenopathy and the lymphoid neoplasms associated with the acquired immune deficiency syndrome. In: Knowles D, ed. *Neoplastic Hematopathology*, 2nd ed. Philadelphia: Lippincott Williams and Wilkins; 2001.
30. Isaacson PG, Campo E, Harris NL. Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease. In: Swerdlow SH, Campo E, Harris NL et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2008:258–59.
31. Ultmann J. Malignant effusions. *CA Cancer*. 1991;41:166–79.
32. Clare N, Rone R. Detection of malignancy in body fluids. *Lab Med*. 1986;17:147–50.
33. Kendall B, Dunn C, Solanki P. A comparison of the effectiveness of malignancy detection in body fluid examination by the cytopathology and hematology laboratories. *Arch Pathol Lab Med*. 1997;121:976–79.
34. Wittchow R, Laszewski M, Walker W et al. Paranuclear blue inclusions in metastatic undifferentiated small cell carcinoma in the bone marrow. *Mod Pathol*. 1992;5:555–58.
35. Odom L, Wilson H, Jamieson B et al. Significance of blasts in low cell count cerebrospinal fluid specimens from children with acute lymphoblastic leukemia. *Cancer*. 1990;66:1748–54.
36. Li CY, Yam LT. Blast transformation in chronic myeloid leukemia with synovial involvement. *Acta Cytol*. 1991;35:543–45.
37. O'Connell JX. Pathology of the synovium. *Am J Clin Pathol*. 2000;114:773–84.
38. Judkins S, Cornbleet PJ. Synovial fluid crystal analysis. *Lab Med*. 1997;28:774–79.
39. Rabinovitch A, Cornbleet J. Body fluid microscopy in US laboratories: data from two College of American Pathologists surveys, with practice recommendations. *Arch Pathol Lab Med*. 1994;118:13–17.
40. Lu J, Gronowski AM, Eby C. Lamellar body counts performed on automated hematology analyzers to assess fetal lung maturity. *Lab Med*. 2008;39:419–23.
41. Besnaed AE, Wirjosoekarto SA, Broeze KA et al. Lecithin/sphingomyelin ratio and lamellar body count for fetal lung maturity: a meta-analysis. *Eur J Obstet Gynecol Reprod Biol*. 2013.
42. Szallasi A, Gronowski AM, Eby CS. Lamellar body count in amniotic fluid: a comparative study of four different hematology analyzers. *Clin Chem*. 2003;49:994–97.
43. Grenache DG, Beshiri A, Gronowski AM et al. Assessment of fetal lung maturity by the lamellar body count; approved guideline. *Clin Lab Stans Institute C58-A*. 2011:31.
44. Lockwood CM, Crompton JC, Riley JK et al. Validation of lamellar body counts using three hematology analyzers. *Am J Clin Path*. 2010;134:420–28.
45. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th ed. Geneva: World Health Organization; 2010.



31 Primary Hemostasis

BARBARA A. O'MALLEY, M.D.

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *hemostasis*, *blood coagulation*, and *thrombosis*.
2. Explain the general interaction of the systems involved in maintaining hemostasis.
3. Distinguish the events that occur in primary hemostasis from those that occur in secondary hemostasis.
4. Differentiate the primary hemostatic plug from the secondary hemostatic plug.
5. Name the three types of blood vessels and explain the general roles of the vasculature and normal endothelial cells in aiding and preventing the activation of the hemostatic system.
6. Identify and define the steps in the normal sequence of events of platelet activation following injury to the endothelium.
7. Describe the role of the primary hemostatic plug in the cessation of bleeding.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Identify key histological features of each type of blood vessel, and explain the metabolic functions of endothelial cells in hemostasis.
2. Identify three key substances stored in platelet dense α-granules and five key substances stored in platelet α-granules (αGs), and explain the role of each in hemostasis.
3. Outline steps platelets undergo in forming the primary hemostatic plug, including the biochemical mediators necessary for platelet adhesion, platelet aggregation, and platelet secretion.
4. Correlate various platelet ultrastructural features with their functions.
5. Identify platelet agonists and predict their effect on platelet function.
6. Describe the biochemical roles of the secreted contents of the platelet granules in hemostasis.
7. Correlate activation with changes in the platelet ultrastructure and biochemistry.
8. Describe the roles of the platelet in secondary hemostasis.

Chapter Outline

Key Terms

Agonist
α-granule (αG)
Arachidonic acid (AA)
Clot retraction
Dense granule (DG)
Dense tubular system (DTS)
Glycocalyx
Glycoprotein Ib (GPIb)
Glycoprotein IIb/IIIa complex (GPIIb/IIIa)
Hemostasis
Human platelet antigen (HPA)
Nonthrombogenic
Open canalicular system (OCS)
Platelet activation
Platelet adhesion
Platelet aggregation
Platelet procoagulant activity
Platelet secretion (platelet release reaction)
Primary hemostatic plug
Secondary hemostatic plug
Thrombogenic
Thrombopoietin (TPO)
Thrombosis

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the bone marrow production of blood cells. (Chapters 3, 4)
- Identify the marrow precursor of peripheral blood platelets. (Chapter 9)
- State the normal lifespan of platelets in the peripheral blood. (Chapter 9)

Level II

- Summarize the hierarchy of stem and progenitor cells in the bone marrow and the growth factors that direct the proliferation and maturation of blood cells. (Chapter 4)
- Describe the general biology of integrins and other adhesion molecules. (Chapter 7)
- Summarize the development of megakaryocytes in the bone marrow and the mechanism of release of platelets into the peripheral blood. (Chapter 9)

CASE STUDY

We will refer to this case study throughout the chapter.

Michael, a 20-year-old male with acute lymphocytic leukemia (ALL), is receiving chemotherapy. Two weeks after his second treatment, he notices small reddish-purple spots on his lower legs and ankles. A CBC reveals Hb 9 g/dL; WBC 2×10^9/L; platelets 19×10^9/L.

Consider what could be responsible for the pancytopenia and the potential consequences of this condition.

OVERVIEW

This chapter is the first of a section describing the processes involved when blood clots in response to vascular injury. The actions of the blood vessels and platelets in hemostasis are collectively called *primary hemostasis*; the actions of the protein coagulation factors are called *secondary hemostasis*. This chapter discusses primary hemostasis and describes the physical structure and the functions of the blood vessels in hemostasis. It then discusses the role of platelets in hemostasis, including structure and functions of platelets. Chapter 32 describes secondary hemostasis (fibrin formation) and fibrinolysis. Chapters 33 and 34 discuss the disorders of hemostasis that result in bleeding, and Chapter 35 discusses disorders that result in thrombosis.

INTRODUCTION

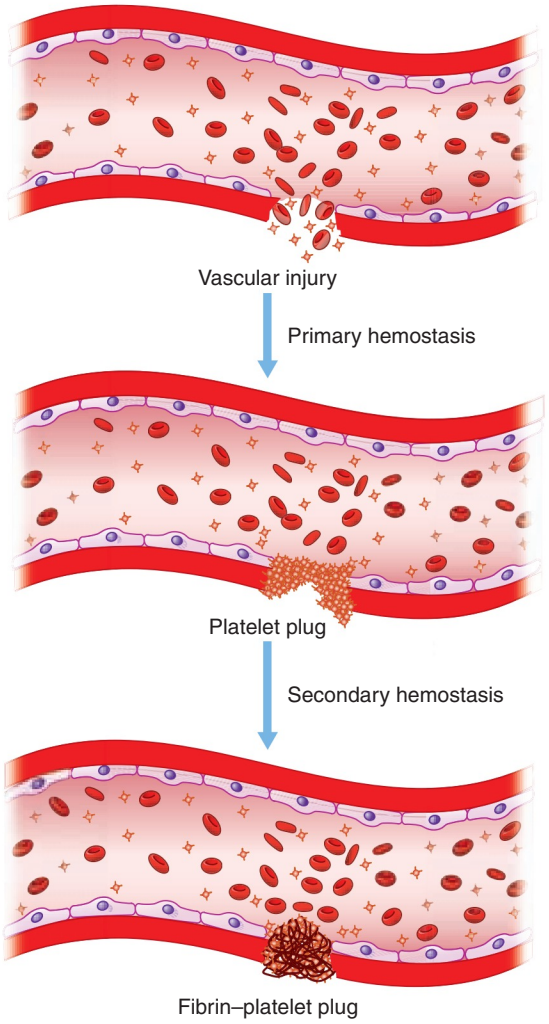
Blood flows through a closed system of vessels called the *circulatory system*. The blood vessels and their constituents are critical in controlling the physiologic functions and integrity of the circulatory system. A traumatic injury, such as a cut to the finger, severs vessels, resulting in bleeding. To minimize blood loss, the normally inert circulating platelets and dissolved plasma proteins mobilize to form a *clot*, an insoluble mass or structural barrier (thrombus), which occludes the injured vessel and prevents further loss of blood. The clot's formation is limited to the area of injury so that normal circulation is maintained in vessels elsewhere in the body. In other words, the hemostatic system is activated when and where it is needed. This system provides a continuous surveillance mechanism that prevents plasma and cells from leaking into the tissues in normal circumstances.

Hemostasis is the property of the circulation that maintains blood as a fluid within the blood vessels under normal circumstances and the system's ability to prevent excessive blood loss upon injury. Hemostasis is from the Greek words *heme*, meaning blood, and *stasis*, meaning to halt. The barrier mass formed to limit blood loss is known as the *hemostatic plug*, *blood clot*, or *thrombus*. Hemostasis requires the interaction of three compartments: the blood vessels, the platelets, and a group of soluble plasma proteins, the coagulation factors. Blood coagulation ("clotting") is the mechanism that transforms the fluid plasma into a gel by converting the soluble protein fibrinogen to the insoluble form, fibrin. Maintaining blood fluidity requires an intact vascular endothelium, quiescent (inactive) blood platelets, and inactive plasma procoagulant proteins. On the other hand, control of bleeding requires rapid activation of platelets and plasma proteins to prevent exsanguination.

Hemostasis occurs in stages called *primary hemostasis*, *secondary hemostasis*, and *fibrinolysis*. During primary hemostasis, the platelets interact with the injured vessels and with one another. This interaction results in a mass of platelets known as the **primary hemostatic plug**, which temporarily arrests bleeding but is fragile and easily dislodged from the vessel wall.

Subsequently, insoluble strands of fibrin become deposited on and within the primary platelet plug to reinforce and stabilize it and to allow the wound to heal without further blood loss. Generation of fibrin constitutes the stage of secondary hemostasis. Fibrin is formed by a series of complex biochemical reactions involving soluble plasma proteins (coagulation factors) as they interact with the injured blood vessels and the platelet plug (Chapter 32). The fibrin-reinforced plug, or thrombus, is called the **secondary hemostatic plug**. The blood has changed from a liquid to a semisolid gel at the site of injury (Figure 31-1 ■).

After the wound has healed, additional components of the hemostatic system break down and remove the clot during the fibrinolytic stage. Physiologic and biochemical inhibitors control all phases and components of the hemostatic system.



■ **FIGURE 31-1** Stages of hemostasis after vascular injury. Bleeding occurs after an injury to a blood vessel. The hemostatic system is activated to prevent excessive blood loss. Hemostasis occurs in two stages: (1) primary hemostasis when the platelets aggregate at the site of the injury and form the platelet plug (primary hemostatic plug) and (2) secondary hemostasis when fibrin develops to strengthen the platelet plug forming the fibrin–platelet plug (secondary hemostatic plug).

★ **TABLE 31-1** Components of Hemostasis

- Vasculature
- Platelets
- Proteins
  - Fibrin-forming proteins
  - Fibrinolytic proteins
  - Inhibitors

Injury also can occur to intact, unsevered blood vessels. In this case, blood clot formation can occur on an interior surface of the damaged vessel wall and result in the abnormal condition **thrombosis**.

In summary, hemostasis occurs because of the interaction of the blood vessels, platelets, and certain plasma proteins (Table 31-1 ★). Plasma proteins involved in hemostasis include those that form fibrin, those that are involved with fibrinolysis, and those that inhibit all stages of the process. Defects in one hemostatic compartment can be overcome by effective utilization of the other two; defects in two of the three compartments generally result in pathologic hemostasis and bleeding.

CASE STUDY *(continued from page 609)*

1. Does Michael have a defect in primary or secondary hemostasis?

ROLE OF THE VASCULAR SYSTEM

The vascular system forms an extensive distribution system for blood, carrying nutrients to all the body's cells and tissues and transporting waste products for disposal. To perform this function, the system must provide an uninterrupted flow of blood, a nonleaking circuit, and maintain blood in a fluid state. The vascular system consists of three types of blood vessels: arteries, veins, and capillaries (Table 31-2 ★). The arteries carry blood from the heart to the capillaries. The veins return blood from the capillaries to the heart. The vessels in which hemostasis occurs are primarily the smallest veins (*venules*) and, to a lesser degree, the smallest arteries (*arterioles*). Venules and arterioles are ~20–200 mcM (μm) in diameter.

Structure of Blood Vessels

The structure of all blood vessels is similar (Figure 31-2 ■), consisting of a central cavity, the lumen, through which the blood flows. The lumen is lined with a continuous, single layer of flattened endothelial cells that separate the blood from the underlying prothrombotic tissues and provide a protective environment for the blood's cellular components. Endothelial cells are attached to a basement membrane that consists of a unique form of collagen embedded in a matrix of adhesive proteins.

★ **TABLE 31-2** Structure and Functions of Blood Vessels of the Microcirculation

Blood Vessel	Structural Characteristics	Functions
Arterioles	20–200 mcM in diameter	Serve as site of hemostatic activity following injury
	Basement membrane	Regulate blood pressure by change in diameter and chemical mediators
	Primary component, smooth muscle cells	Perform endothelial cell hemostatic functions
	Fibroblasts	
	Thicker wall of collagen and extracellular matrix	
Venules	20–200 mcM in diameter	Serve as major site of hemostatic activity following traumatic injury
	Basement membrane	Exchange nutrients, oxygen, and waste products
	Few smooth muscle cells	Regulate vascular permeability
	Few fibroblasts	Perform endothelial cell hemostatic functions
	Primary component, thin layer of collagen and extracellular matrix	
	Minimal elastic fibers	
Capillaries	5–10 mcM in diameter	Provide hemostatic effects by endothelial cell function
	Endothelial cell monolayer, surrounding pericytes	Exchange nutrients, oxygen, and waste products
	Basement membrane	
	No smooth muscle cells	
	No collagen fibers	
	No elastic fibers	

The walls of arteries and veins consist of three layers of tissue that vary in thickness and composition, depending on the size and type of vessel (Figure 31-2a).[1,2] The innermost layer, the tunica intima, consists of a single layer of endothelial cells, the basement membrane, and subendothelial connective tissue that holds them together.

The middle layer, the tunica media, is thicker in arteries than in veins. It consists of an internal elastic lamina, which separates the intima and the media, variable layers of smooth muscle cells, and an external elastic lamina that separates the media from the adventitia. In arteries, smooth muscle cells predominate in the tunica media and are surrounded by loose connective tissue primarily consisting of collagen, reticular fibers, and proteoglycans. The tunica media of veins contains only a few smooth muscle cells and fewer elastin fibers. In response to a variety of physiologic stimuli, the smooth muscle cells of the tunica media can contract and expand, which constricts or dilates the lumen of the vessel.

The tunica adventitia, the outer coat, is composed of collagen bundles and the vasovasorum, a network of microvessels that supplies oxygen and nutrients to the cells of the media and adventitia. A few fibroblasts are embedded in this layer; these fibroblasts synthesize and secrete fibers and other components of the matrix.

The smallest of the blood vessels, the capillary (Figure 31-2b), connects the arterioles and venules. Capillaries collectively compose by far the largest surface area of all types of blood vessels. They are approximately 5–10 mcM in diameter, just large enough for single blood cells to traverse. Capillaries consist of a layer of endothelial cells on an underlying basement membrane and are surrounded by a discontinuous layer of contractile cells called *pericytes*. The endothelial cells communicate with surrounding pericytes through gap junctions.[1] The tissue beneath a capillary's basement membrane is sparse and contains no smooth muscle cells.

Functions of Blood Vessels in Hemostasis

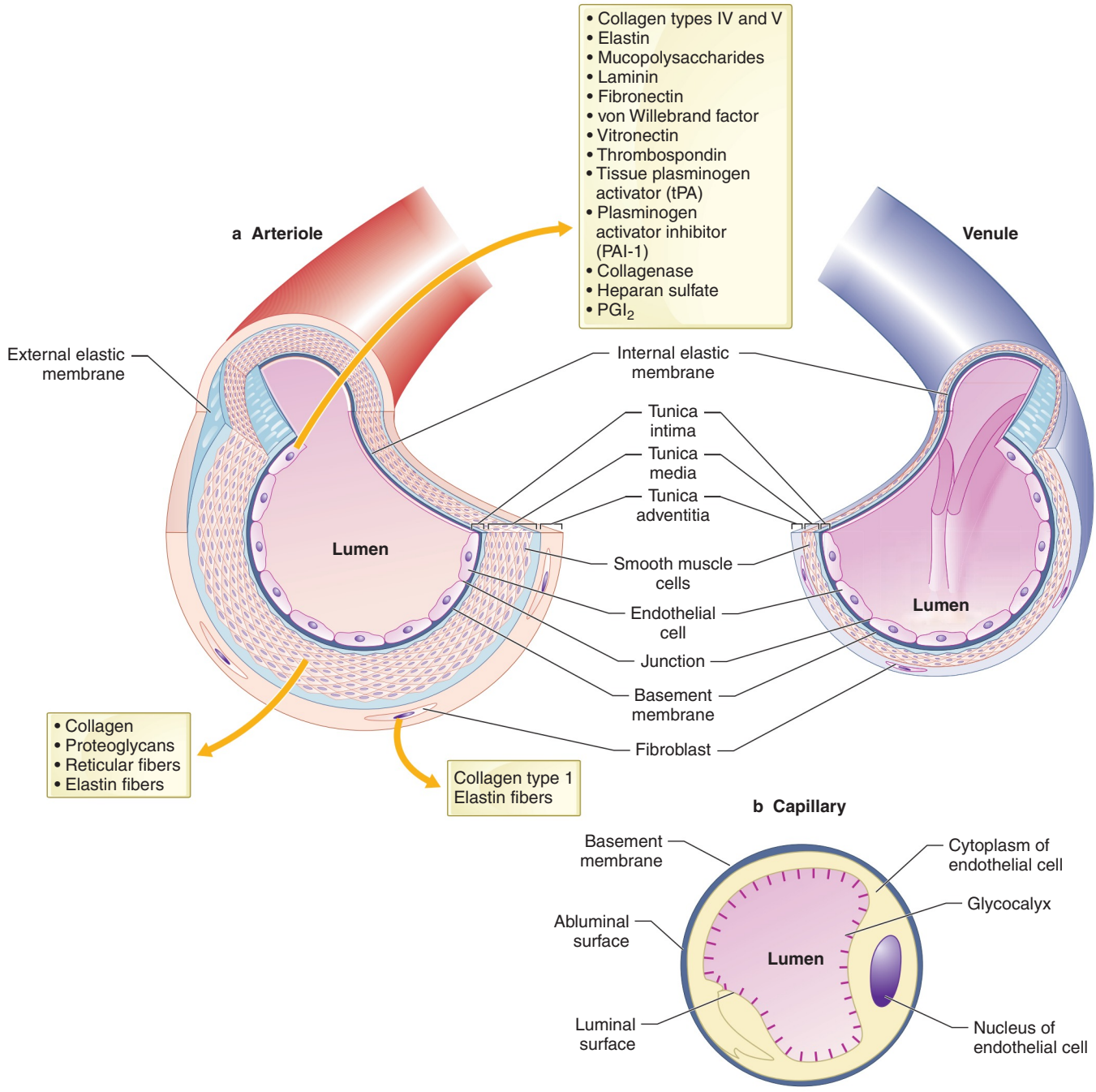
After an injury, the damaged vessels initiate hemostasis. Their first response to injury is constriction or narrowing of the lumen of the arterioles to minimize the flow of blood into the wound area and the escape of blood from the wound site. Vasoconstriction also brings the hemostatic components of the blood (the platelets and the plasma proteins) closer to the vessel wall, facilitating their interactions. Vasoconstriction occurs immediately and lasts a short time.

The mechanism of vasoconstriction is complex. It is caused in part by neurogenic factors and in part by several regulatory substances that interact with receptors on the surface of cells of the blood vessel wall. These include serotonin and thromboxane A_2 (TXA_2), both products of activated platelets, and endothelin-1 produced by endothelial cells.[1]

In contrast, healthy intact endothelial cells synthesize and secrete prostaglandin PGI_2, also called *prostacyclin*, and nitric oxide (NO), also called *endothelium derived relaxing factor (EDRF)*. Both counteract vasoconstriction by causing vasodilation of the arterioles.[3] Both also inhibit activation and recruitment of platelets.

☑ CHECKPOINT 31-1

Think about the last time that you injured your finger with a paper cut. Did your finger bleed immediately? If not, what might have prevented immediate bleeding?



■ **FIGURE 31-2** Structure and function of the blood vessel wall, comparing and contrasting arterioles, venules, and capillaries. **(a)** Arterioles and venules. Endothelial cells, smooth muscle cells, and fibroblasts synthesize and secrete the components of the subendothelial matrix of connective tissue and the basement membrane proteins. The wall of arterioles is primarily composed of smooth muscle cells and contains elastin in contrast to the venule, which has only sparse smooth muscle cells. The media of arterioles is the most prominent layer; the adventitia layer is the largest in venules. **(b)** A capillary consists primarily of endothelial cells and basement membrane with very little connective tissue.

Functions of Endothelial Cells

Endothelial cells lining the blood vessel lumen are the principal components regulating many vascular functions. Some functions modulated by the endothelial cells are hemostatic; others are nonhemostatic in nature (Table 31-3 ★). Endothelial cells play an important role in thromboregulation, a process in which the cells of the vessel wall interact with cells and proteins of the blood to facilitate or inhibit thrombus formation.[3] The endothelial lining of normal, healthy blood vessels is **nonthrombogenic** and antithrombotic, preventing inappropriate clotting. Damaged endothelial cells become **thrombogenic**, promoting the formation of a thrombus or blood clot (Figure 31-3 ■).

Hemostatic functions that inhibit clot formation include providing a nonreactive environment for the components of the hemostatic system (platelets and coagulation proteins) that are inert in the presence of normal endothelium. Both physiologic and biochemical interactions provide this nonreactive environment.[4] Physiologically, the surface of the endothelial cell is negatively charged and repels negatively charged–circulating proteins and platelets. Biochemically, a wide variety of substances are synthesized and secreted by the endothelial cells, which contribute to the nonreactive environment. These include substances that inhibit platelet function as well as coagulation and activate fibrinolysis (Table 31-3):

- *Inhibit platelet function* (PGI_2, NO, and ADPase)
- *Inhibit coagulation* (heparan sulfate/glycosaminoglycans [cofactor for antithrombin]; thrombomodulin [protein C activation]; tissue factor pathway inhibitor [TFPI])
- *Activate fibrinolysis* (tissue plasminogen activator [tPA] and annexin A2 [tPA receptor]; urinary-type plasminogen activator [uPA] and uPA receptor [uPAR])

See Chapter 32 for a discussion of the functions of these components.

Damaged endothelium becomes thrombogenic, producing substances that activate platelets and coagulation proteins and inhibit fibrinolysis. Damaged endothelial cells:

- Produce and secrete von Willebrand factor (VWF) (Chapters 32 and 34), which aids platelets in the initial stage of primary hemostasis
- Produce tissue factor that is released during injury and initiates the formation of fibrin for secondary hemostasis (Chapter 32)

★ **TABLE 31-3** Functions of Endothelial Cells

Component/Characteristic	Function
Hemostatic functions	
Nonthrombogenic	
Negatively charged surface	Repels platelets and hemostatic proteins
Heparan sulfate (HS)	Inhibits fibrin formation (cofactor for antithrombin)
Thrombomodulin (TM)	Binds thrombin, enhances activation of protein C
Endothelial protein C receptor (EPCR)	Binds protein C; facilitates PC activation
Tissue factor pathway inhibitor (TFPI)	Binds TF/FVIIa/FXa complex; inhibits extrinsic pathway of coagulation
Prostacyclin (PGI_2)	Vasodilates; inhibits platelet activation
Tissue plasminogen activator (tPA)	Activates fibrinolytic system
Annexin A2 (tPA receptor) (A2)	Binds tPA and plasminogen; activates fibrinolysis
Urinary-type plasminogen activator receptor (uPAR)	Binds uPA and plasminogen; activates fibrinolysis
Nitric oxide (NO)	Vasodilates; inhibits platelet recruitment and accumulation
ADPase (CD39)	Degrades ADP to AMP and adenosine
Thrombogenic	
Endothelin (ET)	Vasoconstricts
von Willebrand factor (vWF; production and processing)	Carries factor VIII (FVIII) in plasma; facilitates platelet adhesion
Tissue factor (TF)	Initiates fibrin formation, activates FVII
Plasminogen activator inhibitor (PAI-1)	Inhibits activation of fibrinolytic system
Nonhemostatic functions	
Selective blood/tissue barrier	Keeps cells and macromolecules in vessels; allows nutrient and gas exchange
Processing of blood-borne antigens	Contributes to cellular immunity
Synthesis and secretion of connective tissue:	
Basement membrane collagen	Provides backup protection for endothelial cells
Collagen of the matrix	Induces platelet adhesion
Elastin	Vasodilates and vasoconstricts
Fibronectin	Binds cells to one another
Laminin	Contributes to platelet adhesion after injury
Vitronectin	Binds cells to one another; possibly promotes platelet adhesion
Thrombospondin	Binds cells to one another; possibly promotes platelet adhesion

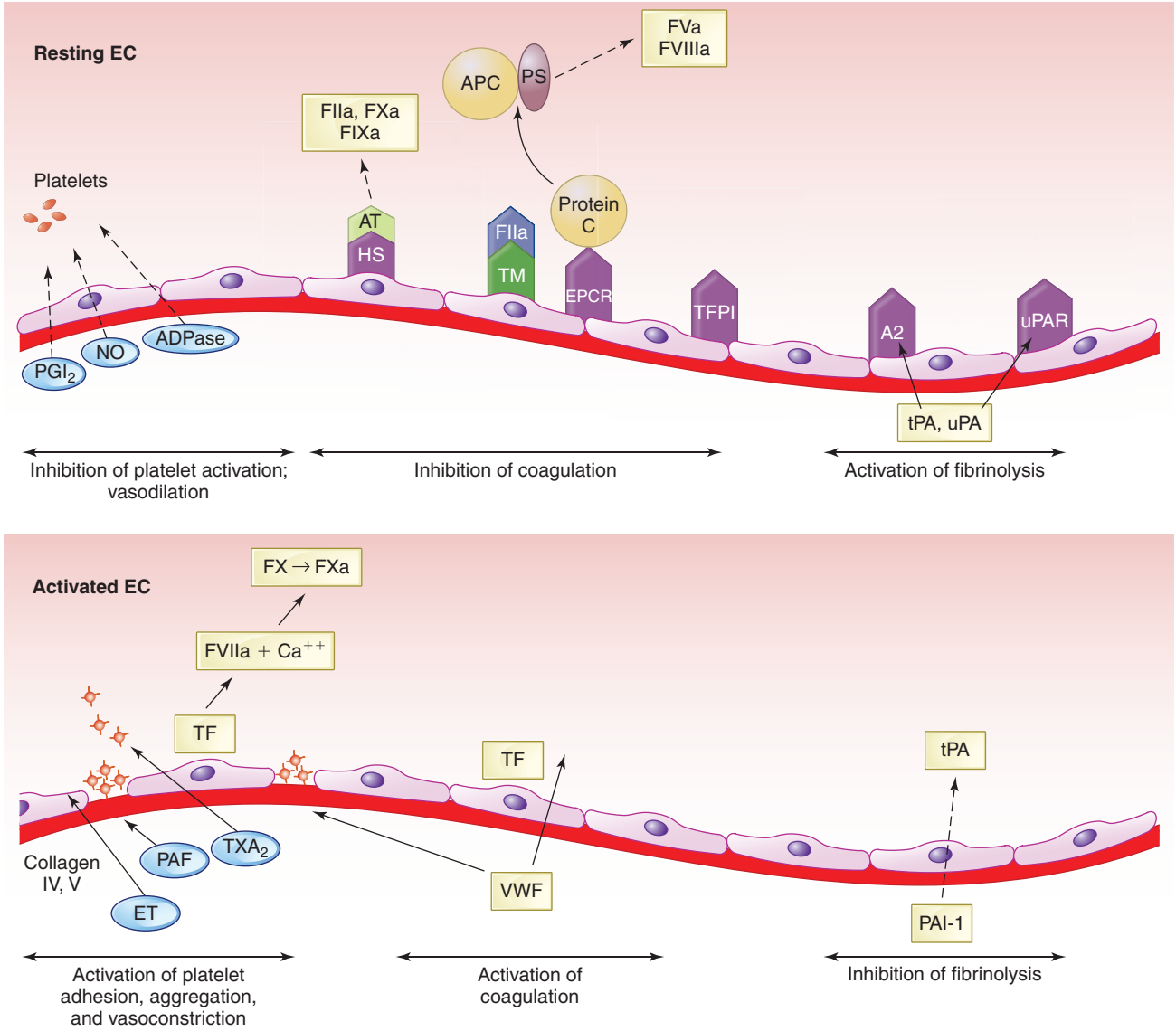


FIGURE 31-3 Antithrombotic characteristics of resting endothelium versus the prothrombotic effects of damaged or activated endothelium. Resting endothelium provides an environment that inhibits activation of hemostasis. This includes secretion of substances that (1) inhibit platelet activation (PGI_2, NO, ADPase); (2) inhibit coagulation (heparan sulfate as a cofactor for AT, TM for activation of protein C, which inactivates activated FVa and FVIIIa; and TFPI); and (3) activate fibrinolysis (tPA, uPA). When endothelium is damaged, it secretes substances that (1) activate platelets (TXA_2, PAF, ET) and bind them to the vessel wall (VWF), (2) activate coagulation (TF, which initiates formation of fibrin), and (3) inhibit fibrinolysis (PAI-1).

EC = endothelial cells; ET = endothelin; PGI_2 = prostacyclin; NO = nitric oxide; HS = heparan sulfate; AT = antithrombin; TM = thrombomodulin; APC = activated protein C; EPCR = Endothelial cell protein C receptor; PS = protein S; TXA_2 = thromboxane A_2; tPA = tissue type plasminogen activator; A2 = Annexin A2 = tPA receptor; uPA = Urinary-type plasminogen activator; uPAR = uPA receptor; PAF = platelet activating factor; TF = tissue factor; TFPI = tissue factor pathway inhibitor; VWF = von Willebrand factor; PAI-1 = plasminogen activator inhibitor-1; → = stimulation; ⇢| = inhibition

- Expose collagen and other adhesive proteins in the subendothelium and secrete platelet-activating factor (PAF), which activate platelets
- Release plasminogen activator inhibitor (PAI-1), which inhibits fibrinolysis. Nonhemostatic functions of the endothelial cells are described in this chapter's Companion Resources

When endothelial injury occurs and bleeding starts, platelets and coagulation proteins in the plasma physically contact exposed subendothelial tissues. Interactions then occur between the vessel wall, platelets, and hemostatic proteins in the plasma to form a blood clot to stop the bleed.

The amount of blood lost from a vessel depends on its size and type as well as the efficacy of the hemostatic mechanism. Hemostasis is most effective in arterioles and venules. Capillaries lack the tissue layers beyond the basement membrane and do not effectively contribute to hemostasis. When larger vessels are severed, hemostatic plug formation takes longer and might not be sufficient to stop bleeding. The pressure within arteries is much higher than in veins, and the blood flow can be so rapid that clotting cannot occur effectively (e.g., within the upper aorta).

☑ CHECKPOINT 31-2

What actions of the endothelial cells prevent clotting from occurring within the blood vessels?

PLATELETS IN HEMOSTASIS

The second major component of the hemostatic system is the blood platelet. Bizzozero first established the platelet's role in hemostasis in 1882.[5] He noted that they were clumped together as part of thrombi in the mesenteric vessels of rabbits and guinea pigs.

As described in Chapter 9, platelets are anucleate fragments of megakaryocyte cytoplasm released into the circulation as proplatelets. They have a life span of 7–10 days in the circulation if they are not consumed in the process of hemostatic activation sooner. Regulation of megakaryocyte differentiation and maintenance of peripheral blood platelet numbers are primarily regulated by **thrombopoietin (TPO)**. Additional cytokines, including stem cell factor (SCF), interleukin (IL)-3, GM-CSF, IL-6, and IL-11 also influence platelet production.

CASE STUDY *(continued from page 610)*

2. If Michael were given TPO, how would you expect his bone marrow and peripheral blood picture to change?

Platelet Structure

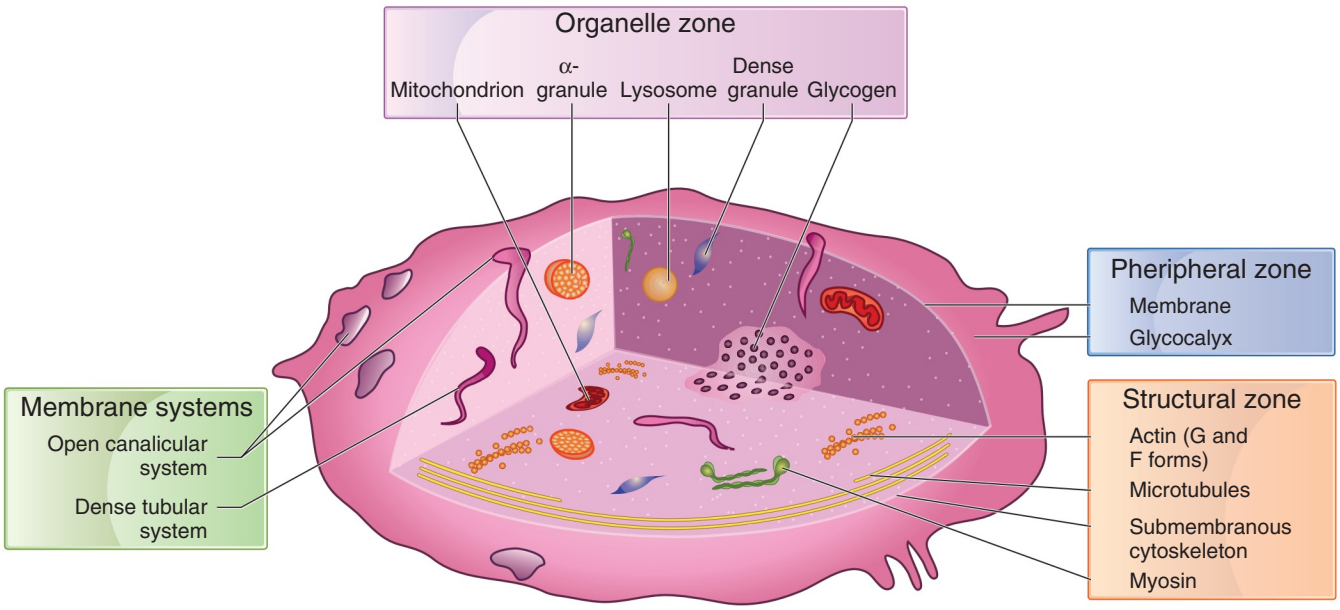
Circulating "resting" (unactivated) platelets are disc-shaped anucleate cell fragments with smooth surfaces. Unlike the exterior surfaces of erythrocytes and leukocytes, the surface membranes of platelets have several openings or pits resembling holes in a sponge. The pits are the openings to membranous channels that extend deep into the interior of the cells.

Circulating platelets repel one another and the surfaces of the endothelial cells that line the interior lumen of blood vessels. After an injury, many changes affecting the platelet morphology and biochemistry occur, causing the platelets to become "activated" after which they interact with the vessel wall and other platelets to form the primary hemostatic plug. To understand the activation process, the ultrastructure of normal platelets must be considered.

The platelet ultrastructure is divided into four arbitrary regions or zones: peripheral zone, structural zone, organelle zone, and membrane systems[6] (Figure 31-4 ■ and Table 31-4 ★). The components of each region have specific functions in activated platelets and are discussed in the following sections.

Peripheral Zone

The platelet's peripheral zone consists of a phospholipid membrane covered on the exterior by a fluffy surface coat and on the interior by a thin submembranous region between the cytoplasmic membrane and the next layer.



■ **FIGURE 31-4** Diagram of platelet ultrastructure.

★ **TABLE 31-4** Platelet Ultrastructure and Functions

Zone and Component	Function/Role
Peripheral zone	**Adhesion and aggregation**
• Glycocalyx	
Proteins, glycoproteins, mucopolysaccharides	
• Phospholipid bilayer	
Phospholipids	Asymmetric arrangement; source of arachidonic acid
• Integral proteins	
• Glycoproteins Ib/IX, IIb/IIIa, GPIa/IIa, GPVI	Adhesion and aggregation
Enzymes	Activation
Structural zone	**Structure and support**
• Microtubules (tubulin)	
• Cytoskeletal network	
• Microfilaments (actin)	
• Intermediate filaments (filamin/actin-binding protein, talin, vimetin)	
Organelle zone	**Storage and secretion**
• Granules	
Dense granules	Nonprotein mediators (ADP, ATP, and serotonin)
α-granules	Protein mediators
Lysosomes	Enzymes
Peroxisomes	Lipid metabolism
• Mitochondria	Oxidative energy metabolism
• Glycogen	
Membrane systems	**Storage and secretion**
• Open canalicular system	Secretion of granule contents
• Dense tubular system	Storage of calcium

Glycocalyx

The surface coat, or **glycocalyx**, is thicker on platelets than on most other cells (~14–20 nm). It consists of glycolipids, membrane glycoproteins, proteins, mucopolysaccharides, and adsorbed plasma proteins, including coagulation factor V (FV), VWF, and fibrinogen (Chapters 32 and 33). The glycocalyx is responsible for the platelet surface's negative charge and is found on the surface membrane of the interior channels as well. The surface negative charge is thought to help prevent platelets from interacting with each other and with negatively charged endothelial cells. Some surface proteins are receptors for substances that cause platelet activation.

Plasma Membrane

The cytoplasmic membrane has a typical trilaminar structure of a bilayer of phospholipids and embedded cholesterol and integral proteins. It functions to maintain cytoplasmic integrity and to mediate interactions between platelets and the vasculature and plasma proteins. The membrane is formed from the demarcation membrane system of the parent megakaryocyte. The surface membrane of platelets invaginates to give rise to an elaborate system of channels, the *surface-connected open canalicular system (OCS)*, which extends throughout the platelet cytoplasm.

An asymmetrical arrangement of the phospholipids is an important factor in the function of platelets. The negatively charged phospholipids (phosphatidylserine [PS], phosphatidylinositol [PI], phosphatidylethanolamine [PE]) are predominately found on the inner half of the bilayer, whereas neutral phospholipids (phosphatidylcholine [PC] and sphingomyelin [SM]) are largely found on the outer half of the bilayer.[7] In resting platelets, the phospholipid asymmetry is maintained by an ATP-dependent amino phospholipid translocase that actively pumps PE and PS from the outer to the inner leaflet.[7] Two other phospholipid-translocating enzymes are found in platelets, an ATP-dependant flippase that slowly transports all phospholipids from the inner to the outer surface and a lipid scramblase that rapidly induces random bidirectional movement of all lipids in the membrane. The negatively charged phospholipids (PS, PE) accelerate several steps in the plasma protein coagulation sequence. The placement of these phospholipids on the platelet's inner membrane separates them from the plasma coagulation proteins and prevents inappropriate coagulation. However, during platelet activation, activation of scramblase causes the movement of these phospholipids to the platelet surface, facilitating their interactions with plasma procoagulant proteins.

The cytoplasmic membrane contains integral proteins that are receptors for stimuli involved in platelet function, including receptors for VWF, fibrinogen, collagen, thrombin, fibronectin, and vitronectin (Table 31-5 ★). Approximately 30 proteins have been identified as platelet membrane glycoproteins, and a nomenclature system has been developed using the acronym GP for glycoprotein and a roman numeral from I to IX according to electrophoretic migration by decreasing molecular weight. Many of these receptors are integrins, heterodimeric proteins identified by their α and β subunits. Those receptors can be referred to by either nomenclature.

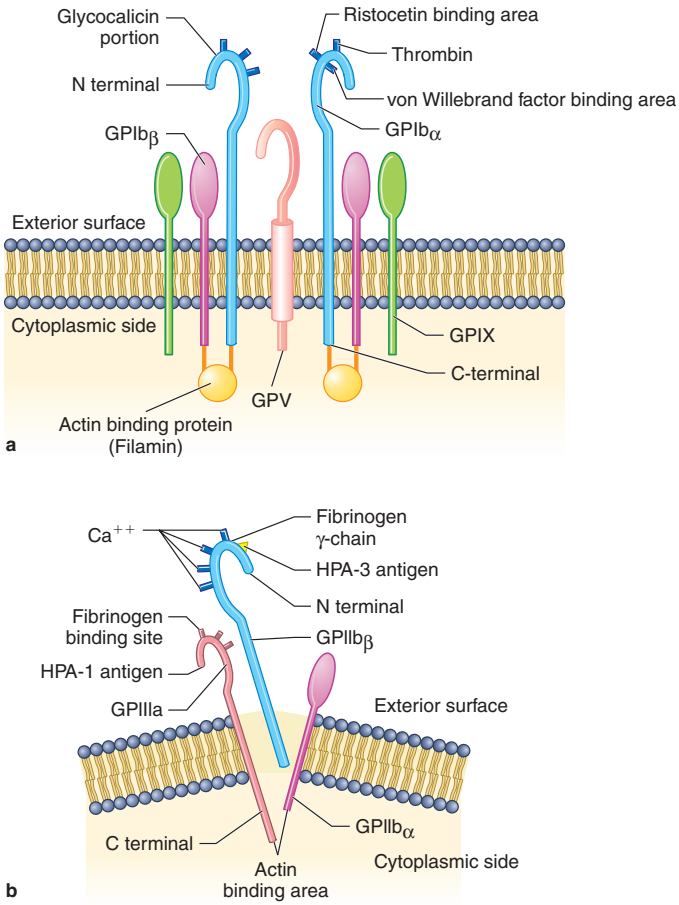
Membrane Receptors

GPIb/IX. **Glycoprotein Ib (GPIb)** is the major platelet receptor for VWF (Figure 31-5a ■).[8] It is found in the platelet membrane as a complex with GPIX and GPV in a ratio of 2:2:1. The function of GPV

★ **TABLE 31-5** Platelet Receptors

Ligand	Receptor(s)
Collagen	GPIa/IIa ($\alpha_2\beta_1$) GPIIb/IIIa, ($\alpha_{IIb}\beta_3$) GPIV GPVI
Fibrinogen	GPIIb/IIIa, ($\alpha_{IIb}\beta_3$) Vitronectin receptor ($\alpha_V\beta_3$)
Fibronectin	GPIc/IIa ($\alpha_5\beta_1$) GPIIb/IIIa ($\alpha_{IIb}\beta_3$)
Thrombospondin	$\alpha_V\beta_3$ GPIV
Vitronectin	$\alpha_V\beta_3$ GPIIb/IIIa, ($\alpha_{IIb}\beta_3$)
von Willebrand factor	GPIb/IX/V GPIIb/IIIa ($\alpha_{IIb}\beta_3$)
Laminin	GPIc/IIa ($\alpha_6\beta_1$)

GP = glycoprotein; integrin nomenclature in parentheses



■ **FIGURE 31-5** Structure of platelet membrane glycoproteins. **(a)** Glycoprotein Ib is composed of an α- and a β-chain. Both span the phospholipid bilayer. The α-chain is larger and contains the binding sites for thrombin, ristocetin, and VWF. Actin-binding protein (filamin) is attached to the cytoplasmic side. Glycoprotein IX and glycoprotein V associate with glycoprotein Ib in the platelet membrane. **(b)** Glycoproteins IIb and IIIa associate in a complex after platelet activation. Binding sites for fibrinogen as well as platelet-specific antigens are present. Cytoplasmic portions of each component have binding areas for actin.

is not known, and no clinical bleeding problems are associated with its absence. GPV is not required for either membrane expression of the GPIb/IX complex or for interaction between GPIb/IX and VWF. The function of GPIX is also unknown, but it is required for efficient surface expression of GPIb. Rare bleeding disorders are associated with abnormalities of GPIb and with GPIX. The complex (also known as *CD42*) is referred to in this text as *GPIb/IX*.

Glycoprotein Ib consists of two polypeptide chains, α and β. The GPIb α-chain is larger and contains the binding sites for VWF, thrombin, and ristocetin (used in the platelet aggregation test described in Chapter 36). Other ligands for GPIb/IX include P selectin, found on activated platelets and endothelial cells, and the integrin $\alpha_M\beta_2$, found on leukocytes. Interactions with these ligands help recruit leukocytes to activated platelets at sites of injury.[9] The binding sites are located on the major extracellular portion of GPIb called *glycocalicin*. The glycocalicin portion of the chain extends from the platelet surface and can be cleaved by proteolytic enzymes including thrombin, ADAM10, and ADAM17. Plasma levels of glycocalicin correlate with platelet production and can be used to differentiate thrombocytopenia resulting from decreased platelet production (low plasma glycocalicin) from that resulting from increased platelet destruction (elevated glycocalicin).[9,10] The remainder of GPIbα is associated with the GPIbβ chain and spans the phospholipid bilayer. On the cytoplasmic side, both the α and β portions are associated with the actin-binding protein filamin. Each platelet contains approximately 25,000 GPIb/IX complexes.[11] GPIb/IX functions in the platelet adhesion process (see the section "Platelet Adhesion").

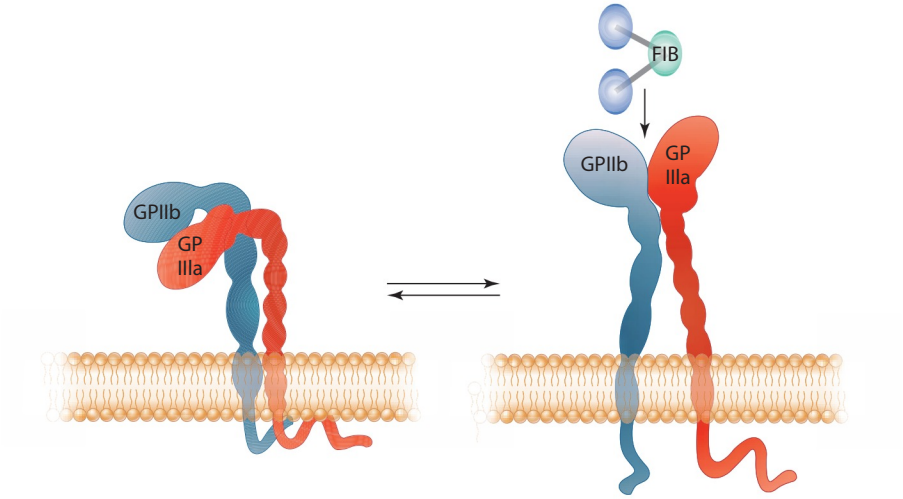
GPIIb/IIIa. Glycoproteins IIb and IIIa associate in the membrane, forming a noncovalently associated **glycoprotein IIb/IIIa (GPIIb/IIIa)** complex, which is the major plasma membrane receptor for fibrinogen (Figure 31-5b). The GPIIb/IIIa complex also binds other subendothelial adhesive proteins such as VWF, thrombospondin, vitronectin, and fibronectin. Each platelet has approximately 80,000–100,000 copies of this receptor on its external membrane, and another 20,000–40,000 copies are present within platelets on the internal membranes of the α-granules, dense granules, and OCS.[12] These internal receptors translocate to the plasma membrane when platelets are activated and undergo secretion.[13] GPIIb/IIIa is an $\alpha_{IIb}\beta_3$ integrin (CD41/CD61).

Glycoprotein IIb, the larger of the two subunits, is a two-chain protein. The α-chain is embedded in the phospholipid bilayer, and the β-chain protrudes from the platelet surface. Glycoprotein IIIa is a single chain polypeptide and is associated with the GPIIb portion that lies within the phospholipid bilayer. The cytoplasmic tails of the two proteins are associated with actin in the platelet cytoskeleton. Like many integrins, the **GPIIb/IIIa complex** in resting platelets is in an inactive form and has low affinity for binding fibrinogen in solution. When agonists activate platelets, the GPIIb/IIIa complex is converted to a high-affinity ligand-binding conformation required for platelet aggregation (described in the section "Platelet Aggregation")[13] (Figure 31-6 ■). The GPIIb/IIIa receptors also seem to be responsible for the uptake of fibrinogen from plasma into megakaryocytes and platelets.

Collagen Receptors. Collagen is one of the most important platelet activators, and several platelet glycoproteins function as collagen receptors. GPIa/IIa (integrin $\alpha_2\beta_1$), like GPIIb/IIIa, must be activated to exhibit high affinity binding to collagen. GPVI also functions as a collagen receptor, and although it has weaker affinity for collagen, it is more effective in activating platelets. It has been proposed that the two receptors work synergistically; GPIa/IIa supports adhesion, and GPVI supports activation.[14] Other platelet membrane proteins that can bind collagen include GPIIb/IIIa and GPIV.[15]

Platelet Alloantigens

Polymorphisms (nucleotide sequence differences) in several of the major platelet glycoproteins have been described. These polymorphisms are immunogenic, and the different inherited alleles thus produce unique alloantigens associated with the platelet surface, designated as **human platelet antigens (HPA)**. Polymorphisms within the β_3 integrin (GPIIIa) polypeptide result in a number of different



■ **FIGURE 31-6** Activation of GPIIb/IIIa. **(a)** Inactive GPIIb/IIIa as found in inert platelets: complex is bent or folded in an inactive conformation. **(b)** Activated form of GPIIb/IIIa: GPIIb-IIIa exists as an "open" or extended conformation.

platelet alloantigens, including HPA-1 (formerly called Pl^A), HPA-4, and others. Polymorphisms within the α_{IIb} integrin (GPIIb) polypeptide result in the HPA-3 platelet alloantigens (formerly called Bak and Lek). Platelet alloantigens have also been associated with polymorphisms of integrin α_2 and GPIbα polypeptides.[16] Studies are ongoing to determine whether these polymorphisms correlate with either bleeding or thrombotic tendencies.[6]

☑ CHECKPOINT 31-3

If a patient inherited a mutation of the gene for glycoprotein IIIa that resulted in its absence, what two platelet antigens would be decreased or absent?

Structural Zone

The structural zone consists of *microtubules* and submembranous and cytoplasmic networks of *microfilaments* and *intermediate filaments*. Functionally, the structural zone supports the plasma membrane, stabilizes the platelet's resting discoid shape, and provides a means of shape change when the platelet is activated.

Microtubules

Resting platelets have a microtubule coil, which completely surrounds the circumference of the platelet just below the plasma membrane, stabilizing the discoid shape of the resting platelet. Microtubules (MT) are the largest of the cytoskeletal filaments (5-nm diameter) and are composed of the protein tubulin.

Microfilaments and Intermediate Filaments

The submembranous protein network consists of structural proteins (intermediate filaments) that form a cytoskeleton supporting the plasma membrane (Web Figure 31-1). Actin, the most abundant protein in platelets, accounts for 15–20% of the total protein content. Upon platelet activation, the submembranous microfilaments and intermediate filaments reorganize, polymerize, and form the characteristic pseudopods seen in activated platelets.

Actin also is part of a network of structural support proteins dispersed throughout the cytoplasm. In the cytoplasm, actin is associated with myosin and several other contractile proteins similar to those of smooth muscle. Unlike smooth muscle, in which the ratio of actin:myosin is about 7:1, the platelet ratio is ~100:1. This network also undergoes significant changes in structure and location when platelets become activated, resulting in the central contraction of MT and the relocation of organelles during platelet activation and shape change.

Organelle Zone

The organelle zone is located beneath the MT layer and consists of mitochondria, glycogen particles (which support the platelet's metabolic activities), and four types of granules dispersed within the cytoplasm: *dense granules*, *α-granules*, *lysosomes*, and *peroxisomes*. They serve as storage sites for proteins and other substances essential for platelet function. Generally, mature platelets have very few ribosomes and little rough endoplasmic reticulum.

Dense Granules

Platelets contain 3–8 electron-dense organelles, each 20–30 nm in diameter. These **dense granules (DGs)** are so named because they appear denser in electron micrographs than the other types of granules because of their high Ca^{++} content. These granules contain mediators of platelet function and hemostasis that are not proteins: ADP, ATP, and other nucleotides as well as phosphate compounds, calcium ions, and serotonin (Table 31-6 ★). The ADP in the DG is known as the *nonmetabolic* or *storage pool* ADP to distinguish it from metabolic ADP found in the cytoplasm. The ratio of ADP:ATP in the DG is higher than that found in the cytoplasm (3:2 versus 1:8 in the cytoplasm).[6] The metabolic pool of ATP/ADP provides energy for normal platelet metabolism, whereas the storage pool is important in platelet activation reactions. Serotonin is taken up from the plasma and stored in the DGs.

α-Granules

The most numerous of the four types of granules are the **α-granules (αGs)**, numbering ~50–80 per platelet. They contain a variety of bioactive substances that can be divided into two major groups

★ **TABLE 31-6** Composition and Functions of Platelet Dense Body Contents

Component	Role
• ADP (nonmetabolic)	Agonist for platelets
• ATP (nonmetabolic)	Agonist for cells other than platelets; activates Ca^{++} influx channel in plasma membrane
• Other nucleotides	Unknown
• Polyphosphates	Acceleration of factor V activation
• Calcium	Platelet activation
• Serotonin	Vasoconstriction; platelet agonist

(Table 31-7 ★): One group consists of proteins similar to hemostatic proteins (coagulation factors, inhibitors) found in the plasma. Some, such as VWF, are synthesized in the megakaryocyte as the platelets develop. Others, such as fibrinogen, immunoglobulins, and albumin, are endocytosed from the plasma by megakaryocytes and packaged in the αG during thrombopoiesis.[17] The second group includes proteins with a variety of functions. Some are found exclusively in platelets (e.g., platelet factor 4 and β-thromboglobulin), and their presence in the plasma can be used as a marker of in vivo platelet activation. αGs contain both proangiogenic proteins (e.g., vascular endothelial growth factor [VEGF]) and antiangiogenic factors (e.g., endostatin).[6] They contain factors that affect the growth and gene expression of smooth muscle cells in the blood vessel wall (e.g., platelet-derived growth factor), which play a major role in vessel repair following injury. Plasminogen activator inhibitor (PAI) is present in platelet αGs in addition to being synthesized by endothelial cells. Platelet αGs also contain a number of adhesive proteins including fibronectin, VWF, vitronectin, and thrombospondin. Platelets contain distinct subpopulations of αGs, which are thought to undergo differential release during platelet activation. For example, fibrinogen and VWF are localized in separate αGs.[6]

★ **TABLE 31-7** Composition and Functions of Platelet α-Granule Proteins

Protein	Role
Group I—hemostatic proteins	
• Fibrinogen	Platelet aggregation
	Conversion to fibrin
• FV, FXI, FXIII	Fibrin formation
• Protein S, tissue factor pathway inhibitor, α_1 protease inhibitor, C1 inhibitor, α_2-macroglobulin	Inhibition of coagulation
• von Willebrand factor	Platelet adhesion
	Carrier of FVIII in plasma
• Plasminogen	Conversion to plasmin (fibrinolysis)
• Plasminogen activator inhibitor-1, α_2-plasmin inhibitor	Fibrinolytic inhibitors
Group II—nonhemostatic proteins	
Platelet specific	
• β-thromboglobulin	Chemoattractant for neutrophils, fibroblasts
	Neutralizer of heparin
• Platelet factor 4	Neutralizer of heparin
	Weak neutrophil and fibroblast chemoattractant
• Multimerin	Complex with factor V
Mitogenic factors	Promotion of regrowth of smooth muscle cells (wound repair)
• Platelet-derived growth factor (PDGF)	
• Transforming growth factor-β	
• Epidermal growth factor	
• Insulinlike growth factor	
• Vascular endothelial growth factor	Angiogenic factor
Miscellaneous	
• Albumin, immunoglobulins	Unknown
• Thrombospondin, fibronectin, vitronectin	Adhesive glycoproteins

Lysosomes and Peroxisomes

Lysosomes contain several hydrolytic enzymes and are similar to the lysosomes found in other cells. Platelets can secrete lysosomal enzymes when activated, but the release is slower and less complete than release from α and dense granules. Thus, release is usually seen only with stronger platelet agonists. With lysosomal secretion, proteins present in lysosomal membranes (lysosome-associated membrane proteins [LAMP-1, LAMP-2, LAMP-3]) appear on the plasma membrane and serve as markers of high-level platelet activation.[18] The peroxisomes are thought to be involved in lipid metabolism.[6]

Platelet Metabolic Activity

Platelets contain all of the necessary enzymes for the glycolytic and tricarboxylic acid cycles and for glycogen synthesis and degradation. About 50% of the platelet's energy (ATP) is derived from the glycolytic pathway, and about 50% is derived from the tricarboxylic acid cycle.

Membrane Systems

The fourth structural zone of the platelet is composed of two systems of membranes: the open canalicular system and the dense tubular system.

Open Canalicular System (OCS)

The surface-connected **open canalicular system (OCS)** is an elaborate, interconnected series of channels leading from the platelet surface to its interior. The OCS originates as invaginations of the plasma membrane and serves several functions in platelets. It provides for both the entry of external substances into the interior of platelets and a route for the release of granule contents to the outside. The OCS also represents an extensive internal store of membranes. Activated platelets undergoing shape change, pseudopod formation, and spreading require a significant increase in surface membrane compared with resting platelets, which OCS membranes provide. Because the OCS membranes contain many of the same glycoproteins as the surface membrane, it also serves as a source for additional plasma membrane glycoprotein receptors during platelet activation.[6]

Dense Tubular System (DTS)

The **dense tubular system (DTS)** originates from the residual endoplasmic reticulum of the megakaryocyte. The DTS channels do not connect with the platelet's surface. The DTS is a storage site for

★ **TABLE 31-8** Platelet Roles in Hemostasis

- Maintenance of blood vessel integrity
- Platelet–platelet interactions (primary hemostatic plug)
- Platelet–coagulation protein interactions (secondary hemostatic plug)
- Aid in healing injured tissue

ionized calcium within platelets analogous to the sarcoplasmic reticulum of muscle cells and releases Ca^{++} when platelets are activated.[19] The concentration of calcium ions within the platelet cytoplasm is important in regulating platelet metabolism and activation. The DTS is also a major site of prostaglandin and thromboxane synthesis.[20]

Platelet Function

Platelets are involved in several aspects of hemostasis, including maintaining vascular integrity, forming the primary hemostatic plug, providing a surface for fibrin generation and secondary hemostatic plug formation, and finally promoting repair of the injured tissues (Table 31-8 ★).

Maintenance of Vascular Integrity

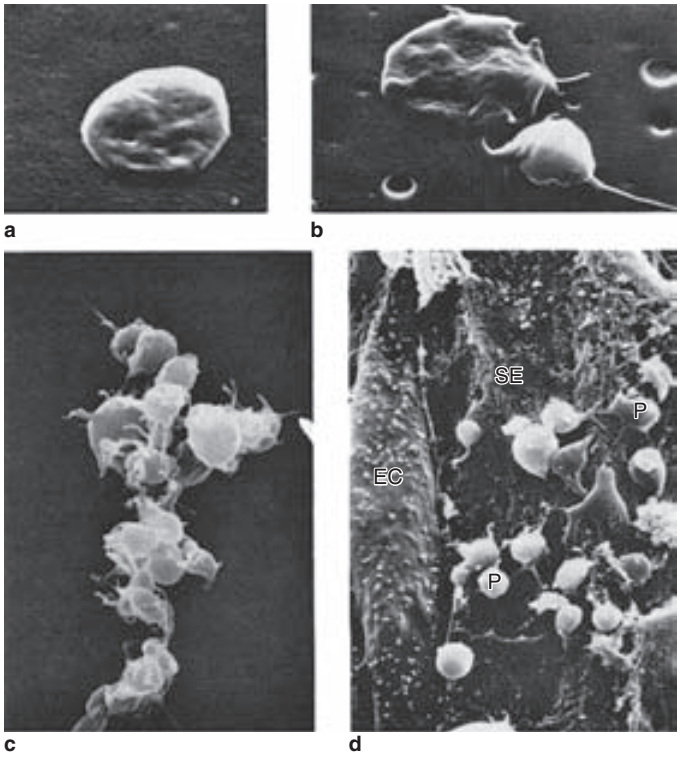
Even in the absence of physical trauma to a vessel, platelets play an important role in maintaining vascular integrity, which requires functional and physical contacts between platelets and the endothelium.[21,22] Platelet granules contain proangiogenic cytokines and growth factors, which are released upon platelet activation. These cytokines function to stabilize an intercellular adhesion protein complex between the vascular endothelial cells. When the circulating platelets are low (thrombocytopenia), the adjacent intercellular junctions disassemble, resulting in leakage of cells and fluid into the surrounding tissue.[21]

Formation of the Primary Hemostatic Plug

When vessel injury occurs, the platelets react by forming the primary hemostatic plug. By sticking first to exposed collagen and other components of the subendothelium and then to each other, the platelets form a mass that mechanically fills defects in the vessel lining and limits the loss of blood from the injury site.

Platelets circulating in blood vessels do not interact with other platelets or other cell types. Circulating platelets are disc shaped and inert in the environment of normal endothelium (Figure 31-7a ■). This is largely the result of endothelial release of prostacyclin (PGI_2) and nitric oxide (NO), which inhibit platelet activation, and the presence of an ADPase (CD39), which degrades ADP, a potent platelet activator.

Injury to the blood vessels causes a change in the normal environment and in response, activation of platelets. The primary hemostatic plug is the result of the transformation of the platelets from an inactive to an active state. The formation of a platelet plug requires several platelet activation events, including adhesion, contraction or shape change, secretion, and aggregation (Figure 31-8 ■).



■ **FIGURE 31-7** Stages of platelet activation. **(a)** "Resting," disc-shaped platelet. **(b)** Partially activated platelet (upper left) and fully activated platelet (spiny sphere—lower right). **(c)** Aggregate of activated platelets. **(d)** Platelets (P) adherent to exposed subendothelium (SE).

EC = intact endothelial cells

Figure courtesy of Dr. Marion Barnhart, Wayne State University School of Medicine.

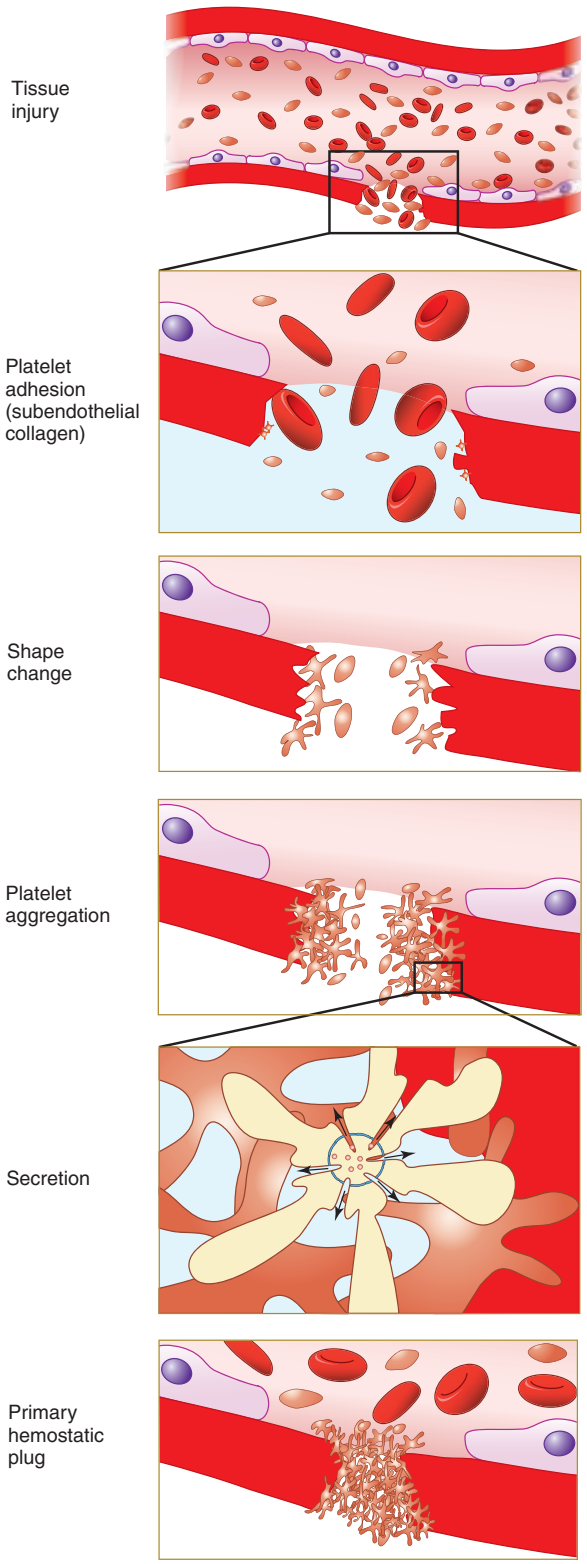


FIGURE 31-8 Diagram of platelets forming the primary hemostatic plug. Tissue injury causes platelets to adhere to subendothelial collagen. Shape change, secretion of granule contents, and aggregation follow. Additional platelets become activated by the secreted substances and clump together, eventually forming a mechanical barrier that halts the flow of blood from the wound.

Platelet Adhesion

The major function of platelets is to seal defects in the vascular network. The initial stimulus for platelet activation is exposure of subendothelial components of the vessel walls that are normally hidden from circulating platelets. The subendothelium contains a large number of adhesive proteins with which the platelets can interact via their specific membrane receptors. The first step in primary hemostatic plug formation, **platelet adhesion**, is the attachment of platelets to collagen and other components of the subendothelium. Platelets contain two collagen receptors: GPIa/IIa and GPVI, which mediate direct collagen binding at low shear rates. Platelet adhesion to collagen at high shear rates, however, requires the presence of VWF and the platelet VWF receptor, GPIb/IX.

Both endothelial cells and megakaryocytes synthesize VWF, which is stored in intracellular organelles, the Weibel-Palade bodies of endothelial cells and αG of platelets. It is secreted into the plasma as well as deposited in the subendothelium where it is bound to collagen (Web Figure 31-2 and Chapter 33). VWF is a multimeric protein made up of a series of identical subunits, each containing binding sites for GPIb/IX on the platelet surface and for collagen in the subendothelium. Circulating (soluble) VWF will not bind to GPIb/IX. However, high shear rates cause conformational changes in immobilized VWF, and platelet adhesion is initiated by interacting with VWF immobilized on collagen[23,24] (Web Figure 31-2). VWF can also bind to the platelet GPIIb/IIIa receptor. Platelet interaction with subendothelial VWF is somewhat transient and insufficient to result in stable adhesion by itself. Firm adhesion requires the synergistic interaction of several platelet receptors. Initial interaction with VWF slows the circulating platelets and facilitates their subsequent interaction with collagen via the GPIa/IIa and GPVI collagen receptors. GPIa/IIa has a higher affinity for collagen, but GPVI is a more effective platelet activator. The result of their interaction is firm adhesion and platelet activation.[11] GPVI activation induces ADP release from the DG and synthesis of TXA_2 from arachidonic acid.[14]

Although the tissues have numerous other adhesion molecules including laminin, vitronectin, and fibronectin and the platelet surface has receptors for these adhesive proteins (Table 31-5), their contributions to platelet adhesion in vivo remain uncertain. Platelets preferentially bind to collagen under conditions of high shear rates.

The GPIb/IX receptor is functionally available in the resting platelet, and binding does not require platelet activation or Ca^{++}. Once its ligand (VWF) becomes available with exposure of the subendothelium, adhesion occurs. Adhesion is a passive, nonenergy-requiring process and is potentially reversible in its early stages. Many platelets adhere in a similar manner until a monolayer of platelets covers the exposed surface of the subendothelium (Figure 31-7d).

Much of what is known about this phase of platelet function has been learned from studying patients with two diseases in which platelets fail to adhere properly: Bernard-Soulier syndrome and von Willebrand disease (VWD). Patients with Bernard-Soulier syndrome have mutations in one of the genes of the GPIb/IX complex that causes either decreased amounts of the receptor complex on the platelet surface or abnormal function of the complex (Chapter 33). Patients with VWD have mutations in the gene encoding VWF (Chapter 34).

☑ CHECKPOINT 31-4

If a patient with Bernard-Soulier syndrome or VWD cut a finger, would you expect bleeding to stop as fast as it does when you cut your own finger? Why or why not?

Platelet Activation

Adhesion of platelets to subendothelial components triggers a series of morphologic and functional changes known as **platelet activation**. *Activation* is a complex process that includes changes in metabolic biochemistry, platelet morphology (shape), surface receptors, and membrane phospholipid orientation. Key activation outcomes are the generation of active GPIIb/IIIa receptors for fibrinogen binding, the secretion of the contents of the platelet granules into the surrounding tissue, and the formation of platelet aggregates. Only activated platelets are able to proceed with the subsequent steps in forming the primary hemostatic plug. Once activated, the platelet response becomes self-perpetuating and irreversible. Through strict control mechanisms, platelet activation remains localized to the injured area. Activation of platelets is summarized schematically in Figure 31-8 and in this chapter's Companion Resources.

Platelet Agonists. A number of substances have been shown to stimulate platelets and to activate them. An agent that induces platelet activation is called an **agonist**. Each agonist binds to the platelet surface at its specific platelet receptor. A signal is transmitted (transduced) internally by the receptor, and a series of reactions in the interior of the platelet leads to subsequent platelet responses. The signal transduction mechanisms in the platelet are similar to those of other cells.

Some agonists (Table 31-9 ★) are generated by the platelets themselves and some by other cells or molecules at the injury site. Some agonists are normally present within cells and are released when the cells are injured or activated; others are synthesized de novo with activation. Agonists are often grouped as "strong" or "weak." Strong agonists (e.g., collagen, thrombin) can activate the full range of platelet functions themselves (shape change, secretion, aggregation). Weak agonists initiate platelet activation but require platelet synthesis and release of endogenous TXA_2 (i.e., activation of the platelet cyclooxygenase pathway) to complete activation through secretion and aggregation. Thrombin is the most potent activator of platelets in vivo.[25]

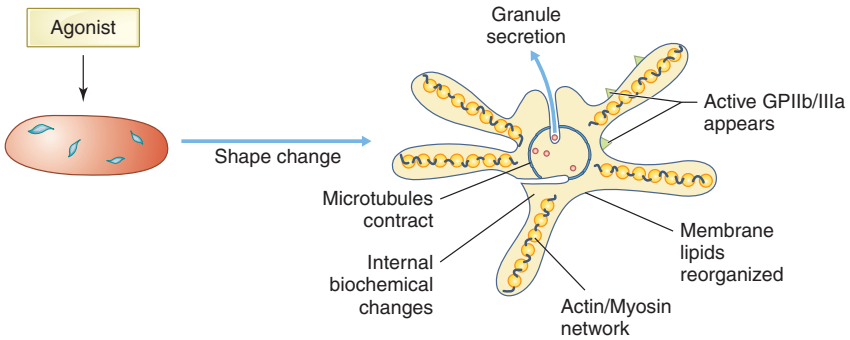
★ **TABLE 31-9** Platelet Agonists and Their Platelet Receptors

Agonists	Agonist Receptors
Platelet-derived agonists	
• ADP	$P2Y_1$, $P2Y_{12}$
• Serotonin	$5HT_{2A}$
• Platelet-activating factor (PAF)	PAFR
• Thromboxane A_2 (TXA_2)	TP
Other (nonplatelet-derived) agonists	
• Collagen[a]	GPIa/IIa, GPVI
• Thrombin[a]	PAR1, PAR4
• Epinephrine	α_2-adrenergic receptors

[a] Strong agonists (full activation of platelet does not require cyclooxygenase activity).

Platelet Shape Change

Activation of platelets by a number of agonists is accompanied by a change in morphology from flattened disc-shaped cells to spiny spheres with long projections from the surface called *pseudopods* (Figures 31-7b and 31-9 ■). Shape change involves reorganization of proteins in the structural zone including the microtubules (MT), submembranous cytoskeletal proteins, and actin and myosin cytoplasmic filaments. The MT coil is dismantled, reorganized, and contracted into the center of the cell, concentrating the platelet organelles in the center and causing the change to a spherical rather than flattened cell.[7] This brings the platelet granules into closer proximity to the OCS, facilitating secretion. The protrusive force for developing pseudopods is the result of actin polymerization and is an energy-requiring process. After platelets adhere to the subendothelium, they undergo variable degrees of "spreading," resulting in the development of broad lamellipodia rather than spikelike pseudopods.[26] As a result of the shape change, each platelet has a larger membrane surface area for biochemical reactions and a larger area for contact with the injured tissue and other platelets.



■ **FIGURE 31-9** Platelet shape change after stimulation by an agonist. Pseudopods develop on the platelet surface and contain a network of actin and myosin; the microtubule circumferential ring contracts; the membrane phospholipids are activated; activated glycoprotein IIb/IIIa receptors appear; internal biochemical changes occur; and granule secretion follows.

The cytoskeletal rearrangements resulting in shape change and spreading alter membrane glycoproteins. GPIb/IX receptors are moved from the platelet surface to the OCS and internalized, whereas active GPIIb/IIIa receptors increase in density on the platelet surface as they are mobilized from αG, DG, and OCS membranes.[27] The result is to convert the activated platelet from an "adhesion" state to an "aggregation" state.

Shape change leads to succeeding responses if the stimulus from the agonists is strong enough. In the absence of a sufficiently strong stimulus, the platelet returns to its original discoid shape.

Platelet Secretion (Release)

Following the centralization of organelles by the contractile process responsible for shape change, platelets begin to discharge granule contents into the surrounding area, a process known as **platelet secretion** or **platelet release reaction**. Secretion is an energy-dependent process requiring ATP. The OCS fuses with membranes of granules that have been centralized deep within the platelets' interior during platelet shape change, and the granule contents are then extruded through the OCS to the platelet's exterior. Alternatively, secretion can occur by direct fusion with the plasma membrane.[7]

Secretion provides a positive feedback mechanism in platelet activation. Some of the substances released from the platelet granules (e.g., serotonin, ADP) function as agonists that stimulate membrane receptors on additional platelets. The binding of secreted ligands to platelet receptors repeats itself, resulting in the recruitment of additional layers of platelets and ultimately the formation of a platelet plug. These positive feedback mechanisms ensure an adequate hemostatic response.

Roles of the Granule Contents. The DGs release their contents into the surrounding tissue (Table 31-6). The DGs release of ADP is considered to be of primary importance because ADP functions as an agonist, producing a positive feedback mechanism in the continued stimulation and recruitment of additional platelets. The ADP receptors important for platelet activation are designated $P2Y_1$ and $P2Y_{12}$.[28] Stimulation by ADP results in the typical response of increased cytoplasmic calcium, more platelet release, and the activation of the fibrinogen receptor GPIIb/IIIa.

DGs release calcium extracellularly with the ADP. This calcium is nonmetabolic and is not involved in the internal stimulation processes. The extruded calcium is believed to provide a high concentration outside the platelets necessary for fibrinogen attachment and for other enzymatic reactions that take place on the platelet exterior surface. Refer to Table 31-6 for other components and their functions.

The αGs contain a wide variety of proteins (see Table 31-7 for a partial list of αG components). Some are specific for the platelet, and others are similar to hemostatic proteins found in the plasma. The platelet-specific proteins include platelet factor 4 (PF4) and β-thromboglobulin (βTG). Both have heparin-neutralizing activity, although βTG does not bind heparin as avidly as PF4. Heparin is an anticoagulant used to treat patients who are hypercoagulable or who are at increased risk of thrombosis (Chapter 35). PF4 performs many actions including chemotactic activity for neutrophils, monocytes, and fibroblasts. βTG, which is actually a family of proteins, also is a chemoattractant for fibroblasts and can promote wound healing.

Thrombospondin constitutes about 20% of the protein released from the platelet αG. It is also synthesized by other cells, including endothelial cells, and is found in the extracellular connective tissue. It may function to help stabilize the aggregated platelets.[11]

Platelets contain a number of growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-β, epidermal growth factor, VEGF, and insulinlike growth factor. All function as mitogens and are thought to contribute to healing of the injured tissue.[7]

FV, VWF, and fibrinogen are proteins in the αG that are similar to hemostatic proteins of the plasma. FV can function as a receptor on the platelet surface for hemostatic proteins (FXa and prothrombin; Chapter 32) and is a cofactor in the process of fibrin formation. Platelets contain ~20% of the FV found in whole blood,[29] which, like fibrinogen, appears to be taken up from plasma.[30] The functions of VWF and fibrinogen have been discussed previously. When released from activated platelets, PAI-1 and α_2-antiplasmin seem to protect newly formed clots from lysis. When released from the platelet granules, these various hemostatic proteins can serve as an additional source of these proteins for secondary hemostasis.

Platelet Aggregation

During platelet adhesion, collagen binding to GPVI triggers intracellular signaling that results in activation of GPIIb/IIIa in the platelet membrane, which then binds soluble fibrinogen. The active GPIIb/IIIa complex appears soon after platelet activation with any agonist. Resting platelets do not express a functional GPIIb/IIIa complex and are unable to bind fibrinogen. This prevents unactivated platelets from aggregating as they circulate in the plasma. With the development of the high-affinity, ligand-binding form of GPIIb/IIIa, the platelet is functionally able to undergo platelet aggregation.[7]

Platelet aggregation is the attachment of platelets to one another (Figure 31-7c). Newly arriving platelets flowing into the area become activated by contact with agonists such as ADP and TXA_2 (released by the initial adherent and activated platelets), products from the damaged tissue and endothelial cells, and thrombin (a procoagulant enzyme generated by tissue factor/FVIIa acting on prothrombin) (Chapter 32). With activation, the new platelets undergo shape change and exposure of their active GPIIb/IIIa sites. The fibrinogen bound to activated platelets serves as a bridge, cross-linking GPIIb/IIIa molecules on two adjacent platelets.

Fibrinogen is able to link two platelets because of its unique molecular structure (Chapter 32). Briefly, it is a dimeric molecule composed of two each of three polypeptide chains: Aα, Bβ, and γ. One fibrinogen molecule attaches to the GPIIb/IIIa receptors on two adjacent platelets via binding sites at the carboxy terminal end of each of the γ-chains (Figure 31-10 ■).[31] The α-chains also contain sequences for platelet binding. Approximately 40,000–50,000 molecules of fibrinogen are bound to each activated platelet. Fibrinogen binding is reversible for a time, but after about 10 to 30 minutes, it becomes irreversible. Ca^{++} is needed for platelet aggregation to occur, whereas platelet adhesion does not require Ca^{++}. Fibrinogen and Ca^{++} are supplied by both the plasma and internal platelet storage sites (αG, DG, and DTS), which provide high concentrations of both constituents in the injured area.

A study of patients with Glanzmann thrombasthenia (Chapter 33) demonstrated the importance of GPIIb/IIIa receptors in platelet

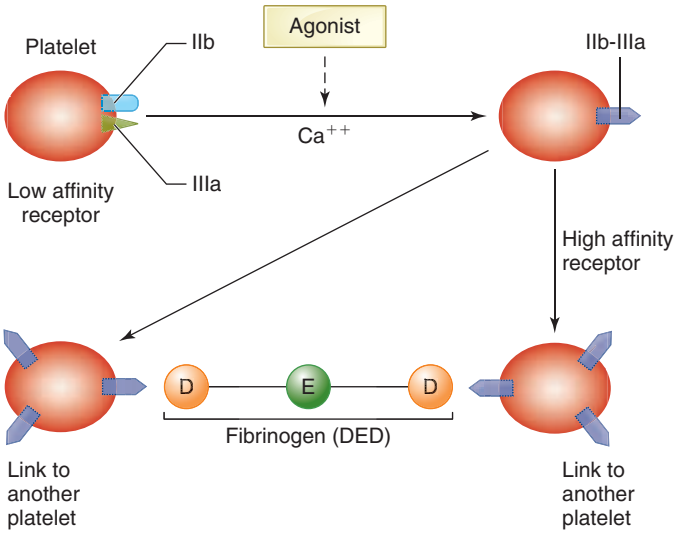


FIGURE 31-10 Platelet aggregation. Agonist stimulation in the presence of Ca^{++} causes high-affinity fibrinogen receptors, activated glycoprotein IIb/IIIa, to appear on the platelet surface. Fibrinogen binds horizontally to two platelets by peptide sequences at the terminal end of its γ-and α-chains in the D domains, one γ-chain to GPIIb/IIIa receptors on each platelet. Fibrinogen thus becomes a bridge between the two platelets.

aggregation. Persons with this rare disease lack functional GPIIb/IIIa receptors, and their platelets do not aggregate in response to various agonists in platelet aggregation tests (Chapter 36). Patients who have decreased levels of fibrinogen have abnormal platelet aggregation as well (Chapter 33). Other adhesive proteins such as VWF, thrombospondin, and fibronectin also bind to the GPIIb/IIIa receptor, but their roles in platelet aggregation in vivo are unclear.

Within in vitro test systems, aggregation occurs in two phases, primary and secondary aggregation (Chapter 36). During primary aggregation, platelets adhere loosely to one another. If the stimulus by agonists is weak, primary aggregation is reversible. Secondary aggregation follows and results in irreversible platelet aggregation. It begins as the platelets start to release their own ADP and other granule contents and to synthesize and release TXA_2 (see the section "Arachidonate Pathway"). The released substances act as additional agonists, which supplement the platelet stimulation. If platelets are unable to release ADP and/or to synthesize TXA_2 secondary aggregation does not occur, and the platelets disaggregate. In general, the in vivo result in such situations increases the time to stop bleeding from a wound.

☑ CHECKPOINT 31-5

Your finger is still bleeding at this point, but the platelets are aggregating to form the primary hemostatic plug. Let's review the key events:

a. To what do platelets first adhere?
b. What bridge and what platelet membrane receptor are needed for platelet adhesion?
c. What bridge and what platelet membrane receptor are needed for platelets to attach to one another?
d. What is the attachment of platelets to one another called?

Biochemistry of Platelet Activation

Platelets become activated after agonists bind to receptors on the platelet surface, initiating signaling events within the platelets. These signaling events eventually lead to the reorganization of the platelet cytoskeleton, granule secretion, and aggregation.

G Proteins. Actions of most of the familiar agonists (collagen, ADP, thrombin, epinephrine, TXA_2, arachidonic acid) and their receptors are linked with guanine nucleotide-binding (G) proteins on the inner leaflet of the platelet phospholipid membrane.[28] G proteins are molecular switches that are inactive when GDP is bound but active after GTP is bound. When the platelet adheres to collagen, G proteins are activated and, in turn, activate enzymes in the platelet membrane and cytoplasm. Some activated enzymes cleave specific membrane phospholipids. The resulting lipid products are "second messengers" that transmit the signal to interior parts of the cells.

Phospholipase C and the Phosphoinositide Pathway. One second messenger pathway important in platelet activation involves activation of the enzyme phospholipase C (PLC) (Figure 31-11 ■). Activated G proteins activate PLC, which cleaves phosphatidyl inositol bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol. PIP_2 is the doubly phosphorylated product of the membrane

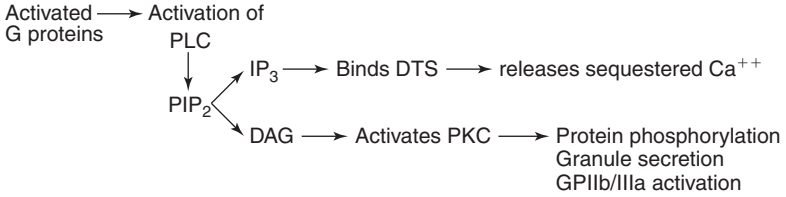


FIGURE 31-11 Phospholipase C/phosphoinositide pathway. Activated G proteins activate PLC. Activated PLC cleaves PIP_2, releasing IP_3 and DAG. IP_3, in turn triggers release of Ca^{++} from the DTS. DAG activates PKC, inducing granule secretion and activation of GP IIb/IIIa, the fibrinogen receptor.

PLC = phospholipase C; PIP_2 = phosphatidyl inositol bisphosphate; IP_3 = inositol triphosphate; DAG = diacylglycerol; DTS = dense tubular system; PKC = protein kinase C

lipid phosphatidyl inositol (PI) produced by PI kinase. The cleavage products released from PIP_2 by PLC function to mobilize calcium ions from storage sites in the DTS (IP_3) and to activate a kinase enzyme, protein kinase C/PKC (diacylglycerol). Activated PKC in turn phosphorylates proteins, contributing to granule secretion and fibrinogen receptor exposure.[28]

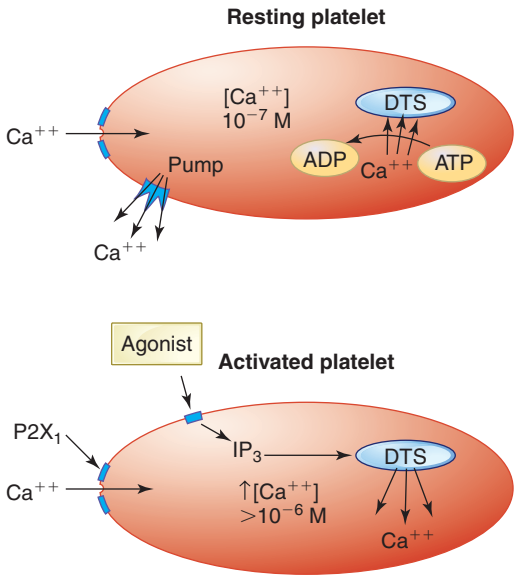
Role of Ca^{++}. Platelet activation is accompanied by an increase in the cytosolic-free Ca^{++} concentration. Ca^{++} serves as an intracellular second messenger and affects enzyme activity and protein–protein interactions. Resting platelets have very low levels of ionic calcium in the cytoplasm (Figure 32-12 ■). Many cellular enzyme systems that are inactive at the Ca^{++} concentration in resting platelets become activated by the increase in Ca^{++} that occurs with platelet activation. These enzymes include phospholipase A2 (PLA_2), phospholipase C (PLC), PI kinase, and myosin light chain kinase ([MLCK], important in the assembly of the contractile mechanism responsible for platelet shape change). The increase in calcium ions results from both the release from internal stores (by IP_3) and the influx from outside the cell, probably via the $P2X_1$ receptor. A direct relationship exists between the amount of cytoplasmic-free calcium and the extent of platelet stimulation.

Arachidonate Pathway. Phospholipase A_2 (PLA_2) is activated by the increase in cytoplasmic calcium and hydrolyzes **arachidonic acid (AA)** from membrane phospholipids. AA serves as a precursor of a variety of regulatory substances, including prostaglandins and leukotrienes. In the platelet, thromboxane A_2 (TXA_2) is synthesized from AA by the enzymes cyclo-oxygenase and thromboxane synthase. In endothelial cells, prostacyclin (PGI_2) is synthesized from AA by cyclo-oxygenase and prostacyclin synthase (Figure 31-13 ■).

TXA_2 is a potent platelet agonist and can stimulate platelet activation and secretion. Platelet activation by "weak" agonists does not produce platelet secretion if TXA_2 synthesis is blocked, which seriously impairs subsequent steps in platelet function. Aspirin (acetylsalicylic acid) irreversibly inhibits cyclooxygenase and prevents affected platelets from synthesizing TXA_2 (Chapter 33). Because aspirin's inhibition of cyclooxygenase is irreversible, the effect lasts for the lifetime of the platelets exposed. TXA_2 is released from activated platelets and enhances vasoconstriction and functions as a platelet agonist, perpetuating the activation process. TXA_2 is a labile compound quickly converted into an inert form, TXB_2, shortly after its synthesis ($T_{1/2}$ ~ 30 seconds).[32]

Cyclic AMP (cAMP) Pathway. cAMP is an important negative regulator of platelet activation (Figure 31-14 ■). cAMP inhibits shape change, platelet secretion, and integrin activation (conversion of GPIIb/IIIa to an active form). PGI_2 from endothelial cells inhibits platelet activation by stimulating adenyl cyclase, increasing cAMP levels. This is one way to limit and localize the formation of the primary hemostatic plug.[7] ADP functions as a platelet agonist by inhibiting adenyl cyclase, thus lowering cAMP levels and permitting platelet activation. The platelet inhibitory drug, dipyridamole, inhibits phosphodiesterase, the enzyme responsible for the degradation of cAMP to AMP, thus stabilizing platelet cAMP levels and inhibiting platelet activation.[28]

Platelet activation and biochemical reactions leading to aggregation are summarized in Figures 31-13 and 31-15 ■.



■ **FIGURE 31-12** Ca^{++} regulation in platelets. Resting platelets maintain low levels of cytoplasmic Ca^{++} via active uptake by the DTS and active extrusion, probably via a Ca^{++} pump. These mechanisms counter a passive diffusion of Ca^{++} into the platelet along a concentration gradient. With activation, platelets increase intracellular cytoplasmic Ca^{++} due to release from the DTS (by IP_3) and increase Ca^{++} influx (probably mediated via ATP and the $P2X_1$ receptor).
DTS = dense tubular system

Platelet Plug

The platelets eventually form a barrier (the primary hemostatic plug) that seals the injury and prevents further blood loss. When the primary hemostatic plug is formed, the bleeding stops. The time for bleeding to cease depends on the depth of the injury and the size of the blood vessel involved. Superficial wounds in which only capillaries and small vessels are affected usually stop bleeding within 10 minutes.

☑ CHECKPOINT 31-6

Your finger has now stopped bleeding. Outline the steps of primary hemostasis that have occurred.

CASE STUDY (continued from page 615)

3. The physician explained to Michael that the reddish-purple spots on his legs and ankles were tiny pinpoint hemorrhages into the skin. Explain the relationship of these hemorrhages to the platelet count.

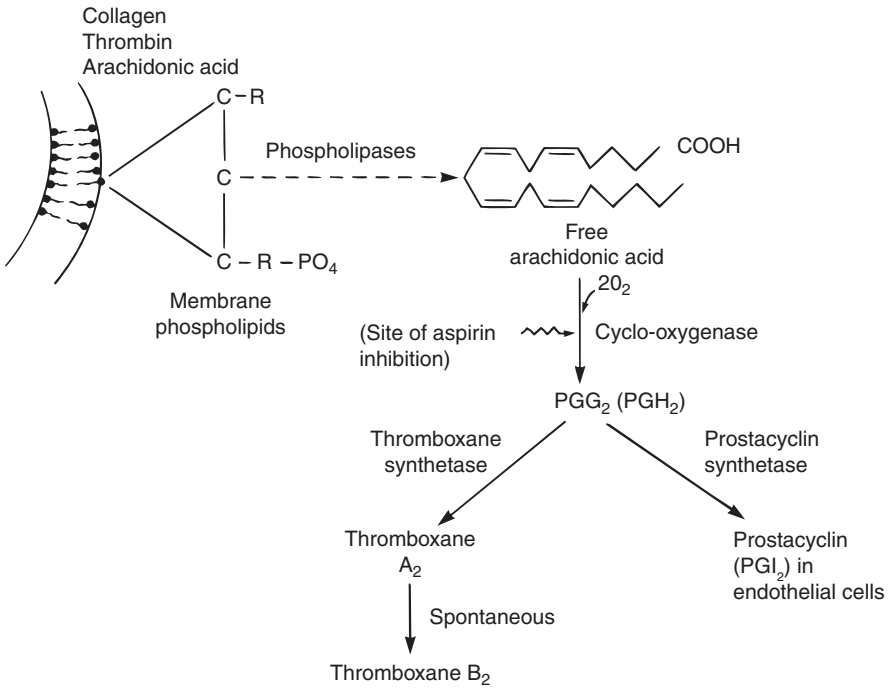


FIGURE 31-13 Biochemical pathways of TXA_2 formation in the platelet. Stimulation of platelet membranes (both intracellular granule and cytoplasmic membranes) by agonists (e.g., collagen, thrombin, arachidonic acid) liberates arachidonic acid from membrane phospholipids. Cyclo-oxygenase incorporates two molecules of oxygen, forming prostaglandin PGG_2 (PGH_2 in the reduced form). Thromboxane synthetase converts PGG_2 to TXA_2, and TXA_2 is spontaneously converted to inactive thromboxane B_2. Alternatively, PGG_2 can be converted to PGI_2, a powerful platelet inhibitor, in endothelial cells.

TXA_2 = thromboxane A_2; PGI_2 = prostacyclin

Platelets and Secondary Hemostasis

The final aspect of platelet activation involves the changes in the platelet membrane that allow platelets to function in secondary hemostasis. Activated platelets accelerate thrombin formation, a function known as **platelet procoagulant activity**. Fibrin-forming proteins (coagulation factors) bind to the surface of activated platelets by binding either to specific receptors on platelets or nonspecifically to negatively charged phospholipids.[33] The resulting platelet-coagulation factor interactions result in formation of the secondary hemostatic plug.

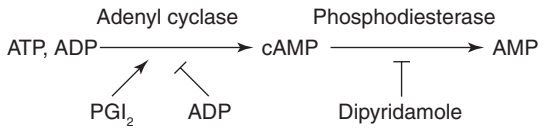


FIGURE 31-14 Role of cAMP in platelets. cAMP is a negative regulator of platelet function, inhibiting various steps of platelet activation. Adenyl cyclase converts ATP or ADP to cAMP. Subsequently, phosphodiesterase degrades cAMP to inactive AMP. PGI_2, a platelet-activation antagonist, activates adenyl cyclase, whereas ADP, a potent platelet agonist, inhibits adenyl cyclase. Dipyridamole, a platelet inhibitory drug, functions by inhibiting phosphodiesterase, thereby stabilizing cAMP and preventing platelet activation.

cAMP = cyclic AMP; → = stimulation; ⊣ = inhibition

The primary platelet plug is relatively unstable and is easily dislodged. During secondary hemostasis, fibrin forms between and around the aggregated platelets. Platelets enhance the fibrin-forming processes by mechanisms explained in Chapter 32. The proteins that interact enzymatically to form fibrin must be assembled on a lipid surface where the reactions take place. This localizes coagulation and the formation of fibrin to the developing thrombus. It also protects coagulation enzymes from inactivation by inhibitors. The membrane phospholipids of activated platelets are the primary source of this lipid surface.

Fibrin-forming proteins do not bind to resting platelet surfaces in the circulation. In resting platelets, the negatively charged phospholipids (PS and PE) are almost exclusively found in the inner half of the membrane bilayer. During the activation of platelets, the membrane phospholipids flip-flop, and the negatively charged phospholipids move to the outer leaflet via a Ca^{++}-activated scramblase enzyme that reverses the asymmetric distribution of phospholipids.[25] This phospholipid rearrangement provides the phospholipid surface, allowing coagulation factors to bind, become activated, and initiate fibrin formation. *Platelet factor 3* activity is an older name for this platelet procoagulant activity. The fibrin-stabilized platelet plug is the secondary hemostatic plug.

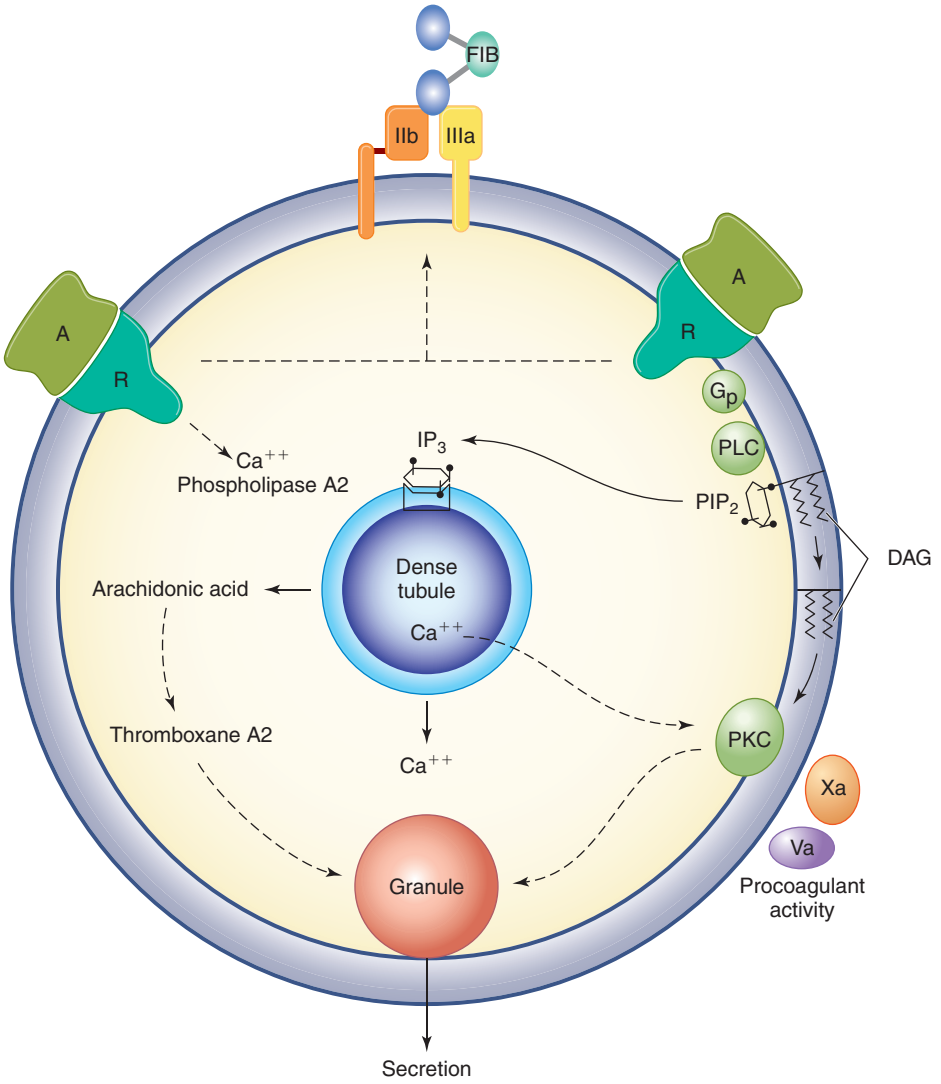


FIGURE 31-15 Schematic diagram showing platelet biochemical changes after activation. An agonist (A) binds to a receptor (R) on the platelet. The GPIIb/IIIa complex appears soon after platelet activation with an agonist. Gp on the inner part of the phospholipid membrane (linked with R) becomes activated and in turn activates PLC, which cleaves PIP_2 to form IP_3 and DAG. Subsequently, Ca^{++} is mobilized into the cytoplasm from the DTS. Many enzymes are activated by the increase in Ca^{++}, including phospholipase A2. Arachidonic acid released from the membrane phospholipids is converted to thromboxane, which stimulates platelet secretion. DAG activates PKC, which contributes to granule secretion. Coagulation factors (e.g., FXa, FVa) bind to the platelet surface leading to fibrin formation.

IIb, IIIa = GPIIb/IIIa receptor; FIB = fibrinogen; DAG = diacylglycerol; Gp = guanine nucleotide binding protein (G protein); IP_3 = inositol-3-phosphate; PLC = phospholipase C; PKC = protein kinase C; PIP_2 = phosphatidylinositol bisphosphate

Recent evidence suggests that only a subpopulation of activated platelets actually produces platelet procoagulant activity. Platelets that are simultaneously stimulated with thrombin and collagen show higher levels of membrane-bound activated coagulation proteins than are observed with only a single agonist (called *coated platelets*). Only ~30% of the total platelet population are coated platelets with optimal agonist activation, suggesting they may be the subpopulation of platelets that actually provides the procoagulant surface for secondary hemostasis to occur.[7,34]

The entire platelet–fibrin mass then contracts to a firmer, more cohesive clot. This contraction is called **clot retraction**. An adequate number of functional platelets are needed for clot retraction, a process that can be observed in a test tube of blood when no anticoagulant is added. After blood is placed into the test tube, the formation of fibrin

results in a network or mesh of fibrin strands that extends throughout, trapping essentially all of the blood cells with some serum. The mass of fibrin and trapped cells contracts over a period of 2–24 hours, extruding much of the serum from the fibrin mass.

Clot retraction is believed to occur by the association of adjacent platelet pseudopods with each other and the fibrin strands. Actin and other contractile proteins within the pseudopods cause the platelets to retract. A comparable phenomenon is believed to occur in vivo, consolidating the thrombus into a cohesive mass of platelets and fibrin that seals the wounded vessel and prevents further blood loss. Contraction can also decrease thrombolysis efficiency and enhance wound healing.[35] The stabilized platelet–fibrin mass remains in place until fibroblast repair of the wound results in permanent healing, and fibrinolysis dissolves the mass.

Physiologic Controls of Platelet Activation

Several inhibitory mechanisms balance platelet activation and prevent excessive platelet deposition (Table 31-10 ★). The platelets' contact with agonists is minimized because of the endothelial cell (EC) barrier. The flowing blood produces a dilutional effect, removing platelet agonists, loosely associated platelets, and coagulation proteins from the developing platelet aggregate. ECs produce NO and PGI_2, two potent inhibitors of platelet activation. ECs also have ADPase that cleaves ATP and ADP to AMP, limiting the agonist effects of ADP. Physiologic controls that limit thrombin activity (Chapter 32) also limit platelet aggregation because thrombin is a potent platelet activator. Many agonists have short half-lives that limit their effects, and platelets can become desensitized to stimulation by some agonists. Intraplatelet calcium levels are tightly controlled as is the availability of the active form of the platelet receptor GPIIb/IIIa.

★ **TABLE 31-10** Physiologic Controls of Platelet Activation

• Minimal platelet contact with agonists (endothelial cell barrier)
• Dilutional effect of flowing blood
• Limited platelet responsiveness to agonists (endothelial cell production of NO; $PGI_2 \rightarrow \uparrow$cAMP; ADPase; antithrombin)
• Limited duration of agonist receptor activity (short half-life of agonists)
• Maintenance of tight controls on cytosolic [Ca^{++}]
• Inability of "resting" GPIIb/IIIa to bind fibrinogen

CASE STUDY *(continued from page 625)*

4. What is the most likely cause of Michael's pancytopenia?
5. Why would the administration of growth factors such as erythropoietin (EPO) and TPO be considered in this case?

Summary

After an injury, blood clots as the result of a series of complex biochemical reactions called *hemostasis*. Its purpose is to temporarily re-establish continuity of injured vessels to minimize loss of blood. The components of hemostasis are found in the plasma and in the tissues that compose the blood vessel walls. All of the components are inert until activated by vessel injury.

Hemostasis occurs in phases called *primary hemostasis*, *secondary hemostasis*, and *fibrinolysis*. This chapter discussed the primary hemostasis phase during which the blood vessels and the platelets cooperatively form an aggregate of platelets that mechanically fills the openings in the injured blood vessels and stops bleeding from the wound site. The injured blood vessels contribute by constricting and secreting various biochemical mediators that affect all of the subsequent steps of hemostasis.

The roles of the platelets are to adhere to the injured areas of the blood vessel walls and to aggregate by attaching to one another and by secreting substances stored in the platelet granules. The secreted substances help to attract and activate new platelets that are added to the aggregate and that help the growth of new tissue to permanently heal the wound. The surface of the aggregated platelets is required for the reactions of secondary hemostasis. Several inhibitory mechanisms including blood flow, which dilutes platelet agonists and coagulation proteins, and inhibitors of platelet activation produced by endothelial cells control platelet activation and formation of platelet plugs.

Review Questions

Level I

1. The definition of hemostasis includes the: (Objective 1)
   A. process of maintaining the body temperature
   B. termination of bleeding following a traumatic injury
   C. process of forming a hematoma
   D. regulation of kidney function
2. Which of the following is the primary element that prevents blood from clotting inside blood vessels? (Objective 5)
   A. fibrinogen
   B. arteriole
   C. endothelial cells
   D. platelets
3. What is the action of the blood vessels in hemostasis immediately after an injury? (Objective 5)
   A. thrombosis
   B. aggregation
   C. vasoconstriction
   D. vasodilation
4. Which of the following has happened when a cut finger initially stops bleeding? (Objectives 6, 7)
   A. vasoconstriction of vessels proximal to the cut
   B. vasodilation of vessels distal to the cut
   C. formation of the primary hemostatic plug by aggregated platelets
   D. completion of fibrin formation and formation of the secondary hemostatic plug
5. What is the first step in platelet function after an injury? (Objective 6)
   A. fibrin formation
   B. release of ADP
   C. platelet aggregation
   D. platelet adhesion to collagen
6. What is the process of platelets binding to one another? (Objective 6)
   A. platelet secretion
   B. fibrin formation
   C. platelet adhesion
   D. platelet aggregation
7. Each of the following is involved in hemostasis *except*: (Objective 2)
   A. vasoconstriction by the blood vessels
   B. adhesion and aggregation by the platelets
   C. fibrin formation by proteins in the plasma and platelets
   D. regulation of blood pressure
8. An abnormal condition in which a blood clot forms on the interior of blood vessel is known as: (Objective 1)
   A. secondary hemostasis
   B. thrombosis
   C. primary hemostasis
   D. fibrinolysis
9. The generation of fibrin by a series of reactions of procoagulant proteins is called: (Objective 3)
   A. secondary hemostasis
   B. primary hemostasis
   C. fibrinolysis
   D. hemolysis
10. The formation of a primary hemostatic plug results from reaction between: (Objective 4)
    A. platelets and subendothelial tissue
    B. platelets and coagulation proteins
    C. coagulation proteins and subendothelial tissue
    D. coagulation proteins and VWF

Level II

1. What are the cells that line the central cavity of all blood vessels and related tissues called? (Objective 1)
   A. epithelial cells
   B. endothelial cells
   C. capillaries
   D. smooth muscle cells
2. Platelet dense granules are storage organelles for which of the following that are released after activation? (Objective 2)
   A. calcium, ADP, and serotonin
   B. fibrinogen, glycoprotein Ib, and VWF
   C. ADP, thromboxane A_2, and FV
   D. lysosomal granules, ATP, and FVIII

3. Which of the following is the platelet receptor needed for platelet adhesion? (Objective 3)
   A. glycoprotein IIb/IIIa
   B. actin
   C. VWF
   D. glycoprotein Ib/IX
4. The platelet glycoprotein IIb/IIIa complex is: (Objective 3)
   A. a membrane receptor for fibrinogen
   B. secreted from the dense granules
   C. secreted by endothelial cells
   D. also called *actin*
5. The formation of thromboxane A_2 in the activated platelet: (Objective 6)
   A. is needed for platelets to adhere to collagen
   B. is caused by the α-granule proteins
   C. requires the enzyme cyclooxygenase
   D. occurs via a pathway involving VWF
6. The function of microtubules in the resting platelet is to: (Objective 4)
   A. keep a high level of calcium in the cytoplasm
   B. store and sequester calcium
   C. provide a negative charge on the platelet surface
   D. maintain the disc shape
7. The contents of the platelet granules are released from the platelet: (Objective 3)
   A. through the open membrane system after fusion with the granules
   B. through the microtubules after fusion with the granules
   C. by disintegration of the platelet plasma membrane
   D. by the mitochondria
8. Which of the following is true about the relationship between ADP and platelets? (Objective 5)
   A. ADP is necessary for platelet adhesion.
   B. ADP released from the dense granules is required for adequate platelet aggregation.
   C. ADP is synthesized in the platelet from arachidonic acid.
   D. ADP is released from the platelets' α-granules.
9. The process by which a platelet agonist causes a change in shape of the platelet from a disc to a spiny sphere is known as platelet: (Objective 5)
   A. secretion
   B. aggregation
   C. activation
   D. release
10. Which of the following statements correctly describes the role of cAMP in platelet function? (Objective 7)
    A. Elevated cAMP induces platelet aggregation.
    B. Elevated cAMP inhibits platelet activation.
    C. Elevated cAMP activates cyclooxygenase and TXA_2 production.
    D. Decreased cAMP inhibits platelet activation

Companion Resources

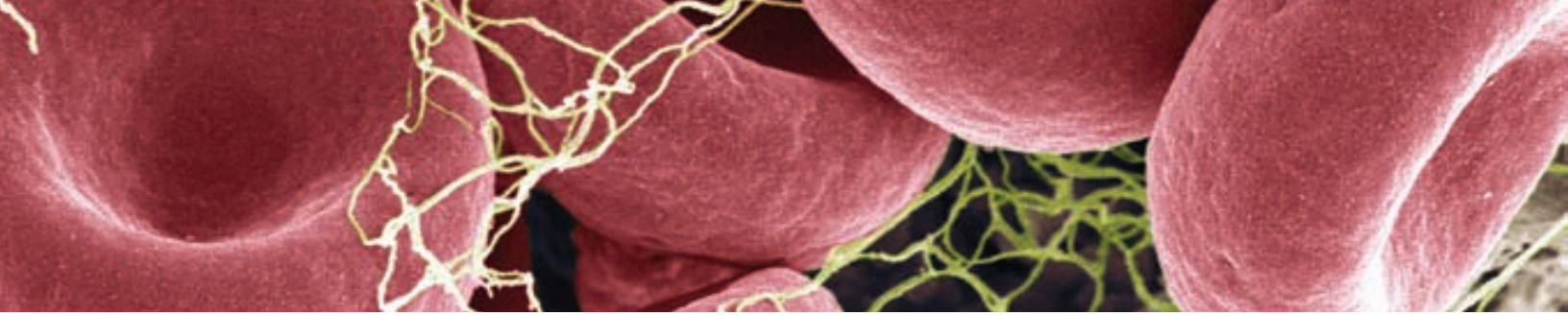
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Karsan A, Harlan JM. The blood vessel wall. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone/Elsevier; 2013:1784–796.
2. Aird WC. Overview of vascular biology. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:493–97.
3. Hajjar KA, Marcus AJ, Muller WA. Vascular function in hemostasis. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010.
4. Krisinger MJ, Conway EM. Role of endothelium in hemostasis. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:569–78.

5. Bizzozero J. Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. *Virchows Arch Pathol Anat*. 1882;90:261–332.
6. Smyth SS, Whiteheart S, Italiano JE et al. Platelet morphology, biochemistry, and function. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010. www.accessmedicine.com/content.aspx?aID=6231303; accessed January 21, 2013.
7. Walsh, PN. The role of platelets in blood coagulation. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:468–74.
8. Berndt MC, Andrews RK. Major platelet glycoproteins: platelet glycoprotein Ib-IX-V. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:382–85.
9. Steinberg MH, Kelton JG, Coller BS. Plasma glycocalicin: an aid in the classification of thrombocytopenic disorders. *N Engl J Med*. 1987;317:1037–42.
10. Beer JH, Buchi L, Steiner B. Glycocalicin: a new assay—the normal plasma levels and its potential usefulness in selected diseases. *Blood*. 1994;83:691–702.
11. Savage B, Ruggeri ZM. The basis for platelet adhesion. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:400–09.
12. Calverley DC, Maness LJ. Platelet function in hemostasis and thrombosis. In: Greer JP, Foerster J, Lukens JN et al., eds. *Wintrobe's Clinical Hematology*, 11th ed. Philadelphia: Lippincott Williams & Wilkins; 2004:651–76.
13. Ye F, Ginsberg MH. Major platelet glycoproteins: integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa). In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:386–92.
14. Watson SP, Farndale RW, Moroi M. Platelet collagen receptors. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:420–30.
15. Abrams CS, Plow EF. Molecular basis for platelet function. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone/Elsevier; 2013:1809–820.
16. Kunicki TJ, Nugent DJ. Platelet glycoprotein polymorphisms and relationship to function and immunogenicity. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:393–39.
17. Cramer EM. Megakaryocyte structure and function. *Curr Opin Hematol*. 1999;6:354–61.
18. Abrams C, Shattil SJ. Immunological detection of activated platelets in clinical disorders. *Thromb Haemost*. 1991;65:467–73.
19. Menashi S, Davis C, Crawford N. Calcium uptake associated with an intracellular membrane fraction prepared from human blood platelets by high-voltage, free-flow electrophoresis. *FEBS Lett*. 1982;140:298–302.
20. Gerrard JM, White JG, Rao GHR et al. Localization of platelet prostaglandin production in the platelet dense tubular system. *Am J Pathol*. 1976;83:283–98.
21. Nachman RL, Rafii S. Platelets, petechiae, and preservation of the vascular wall. *N Engl J Med*. 2008;359:1261–270.
22. Brass LF, Zhu L, Stalker TJ. Novel therapeutic targets at the platelet vascular interface. *Arterioscler Thromb Vasc Biol*. 2008;28:s43–50.
23. Roth GJ. Developing relationships: arterial platelet adhesion, glycoprotein Ib, and leucine-rich glycoproteins. *Blood*. 1991;77:5–19.
24. Andrews RK, Shen Y, Gardiner EE et al. The glycoprotein Ib-IX-V complex in platelet adhesion and signaling. *Thromb Haemost*. 1999;82:357–64.
25. Bennett JS. Overview of megakaryocyte and platelet biology. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:341–48.
26. Hartwig JH, Barkalow K, Azim A et al. The elegant platelet: signals controlling actin assembly. *Thromb Haemost*. 1999;82:392–98.
27. George JN, Pickett EB, Saucerman S et al. Platelet surface glycoproteins: studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest*. 1986;78:340–48.
28. Abrams CS, Brass LF. Platelet signal transduction. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:449–61.
29. Tracy PB, Eide LL, Bowie EJ et al. Radioimmunoassay of factor V in human plasma and platelets. *Blood*. 1982;60:59–63.
30. Camire RM, Pollak ES, Kaushansky K et al. Secretable human platelet-derived factor V originates from the plasma pool. *Blood*. 1998;92:3035–41.
31. Hawiger J. Adhesive ends of fibrinogen and its antiadhesive peptides: the end of a saga? *Semin Hematol*. 1995:32:99–109.
32. Grosser T, Fitzgerald GA. Platelet prostanoid metabolism. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:462–67.
33. Walsh PN. The role of platelets in blood coagulation. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:468–74.
34. Bouchard BA, Butenas S, Mann KG et al., Interactions between platelets and the coagulation system. In: Michelson AD, ed. *Platelets*. San Diego: Academic Press; 2007:377.
35. Kunitada S, Fitzgerald GA, Fitzgerald DJ. Inhibition of clot lysis and decreased binding of tissue-type plasminogen activator as a consequence of clot retraction. *Blood*. 1992;79:1420–27.



32 Secondary Hemostasis and Fibrinolysis

J. LYNNE WILLIAMS, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *hemostasis* and identify the three physiologic compartments involved in the hemostatic mechanism.
2. Differentiate primary and secondary hemostasis.
3. List the coagulation factors using roman numerals and determine how each factor is evaluated in lab testing.
4. Classify the coagulation factors in groups and discuss their characteristics.
5. Evaluate the importance of vitamin K in hemostasis.
6. Describe the mechanism of action of the coagulation proteins.
7. Diagram the sequence of reactions in the coagulation cascade according to the historic concepts of intrinsic, extrinsic, and common pathways.
8. Identify the factors involved in contact activation.
9. Diagram the physiologic pathway of blood coagulation.
10. Define *fibrinolysis*, identify the major components of the fibrinolytic system, and explain why fibrinolysis is a necessary component of hemostasis.
11. List the fragments resulting from fibrinolytic degradation; compare and contrast the fragments resulting from the degradation of fibrinogen and fibrin.
12. List the major biochemical inhibitors that regulate secondary hemostasis.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Explain the interactions of the three physiologic compartments involved in hemostasis.
2. Describe the domain structure of the coagulation factors, determine how this structure affects the action of the serine proteases, and explain the significance of the noncatalytic regions.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

3. Summarize the formation of complexes on a phospholipid surface and explain the significance of these complexes to hemostasis.
4. Integrate the role of contact factors with other systems (complement activation, fibrinolysis, inflammation).
5. Describe the multiple roles of thrombin in hemostasis.
6. Explain the physiologic functions of ADAMTS-13, A2, LRP, EPCR, and uPAR.
7. Compare and contrast physiologic and systemic fibrinolysis.
8. Describe the physiologic controls of hemostasis including blood flow, feedback inhibition, liver clearance, and inhibitors (antithrombin, TFPI, protein C, protein S, and TAFI)
9. Evaluate a case study from a patient with a defect in hemostasis and, using the medical history and laboratory results, determine the diagnosis.

Key Terms

ADAMTS-13
Annexin-II (A2)
Coagulation factor
Common pathway
Contact group
Endothelial cell protein C receptor (EPCR)
Extrinsic pathway
Extrinsic Xase complex
Fibrin degradation product (FDP)
Fibrinogen group
Fibrinolysis
γ-Carboxylation
Intrinsic pathway
Intrinsic Xase complex
LRP receptor (LDL receptor-related protein)
Plasminogen activator (PA)
Plasminogen activator inhibitor (PAI)
PIVKA
Prothrombinase complex
Prothrombin group
Serine protease
Serpin
Thrombin-activatable fibrinolysis inhibitor (TAFI)
Thrombomodulin (TM)
Tissue factor pathway
Tissue factor pathway inhibitor (TFPI)
Tissue-type plasminogen activator (tPA)
Transglutaminase
Urinary-type plasminogen activator (uPA) (urokinase [UK])
UPAR (uPA receptor)
Vitamin K dependent
Zymogen

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Define and summarize the events in *primary hemostasis*. (Chapter 31)

Level II

- Describe the structure of the platelet phospholipid membrane. (Chapter 31)
- Review protein structure, protein domains, and properties of enzymes. (Chapter 2 and previous chemistry and biology courses)

CASE STUDY

We will address this case throughout the chapter.

Shawn, a 10-year-old boy, saw his physician for recurrent nosebleeds and anemia. History revealed that the epistaxis began when he was about 18 months old. The nosebleeds occurred one or two times a month and began spontaneously. His mother reported that he seemed to bruise easily. No lesion was found in the nose. No history of drugs was noted. The family history indicated that the boy's grandparents were cousins. Shawn had a brother who died of intracranial hemorrhage at 10 years of age. Parents and all other relatives showed no bleeding problems.

OVERVIEW

Hemostasis requires the interaction of platelets, blood vessels, and coagulation proteins. The previous chapter described primary hemostasis (platelets and blood vessels). This chapter introduces the plasma protein systems contributing to secondary hemostasis. It begins with an overview of hemostasis and discusses the distinctions between primary and secondary hemostasis. The coagulation proteins are presented in related groups for initial discussion of general properties, mechanisms of action, and protein structure. The chapter describes the functional interactions of these procoagulant proteins from the classical perspective of intrinsic, extrinsic, and common pathways. The current concept of in vivo blood coagulation initiated through a cell-based system is discussed. The chapter presents the fibrinolytic system (components, activators, inhibitors) and defines the products of fibrinolytic degradation. The chapter summarizes the various

components contributing to the control of hemostasis, including physiologic processes (blood flow, liver clearance), positive and negative feedback mechanisms, and natural inhibitors.

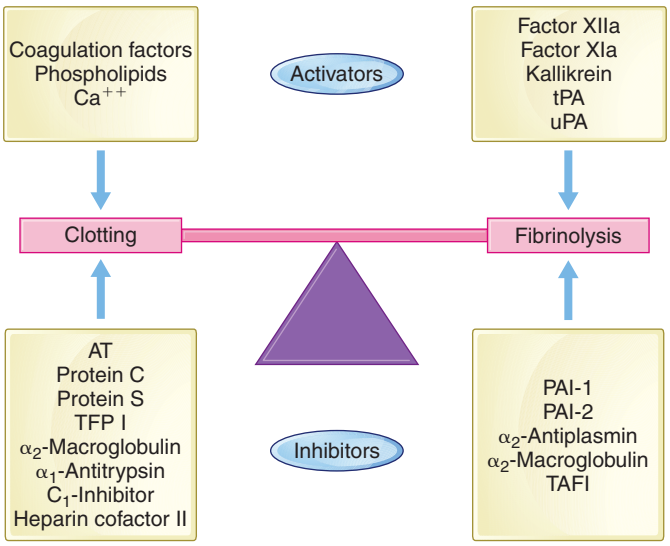
INTRODUCTION

Hemostasis is a carefully balanced process by which the body maintains blood in a fluid state within the vasculature and prevents loss of blood from the vascular system upon injury. Although often thought of as "clot" formation, hemostasis also includes fibrin (clot) dissolution and vessel repair. Hemostasis involves a series of complex and highly regulated events linking platelets, vascular endothelial cells, and coagulation proteins (**coagulation factors**). The interaction of these three components at the cellular and molecular levels determines whether the equilibrium between bleeding (hemorrhage) and clotting (thrombosis) is maintained (Figure 32-1 ■). The delicate balance between bleeding and clotting requires both amplification reactions to ensure sufficient activity when needed and important regulatory reactions to ensure that excess activity does not occur.

As discussed in Chapter 31, hemostasis consists of two stages: primary and secondary. Primary hemostasis (Chapter 31), the formation of the unstable platelet plug, is the first response to vascular injury. Secondary hemostasis, the reinforcement of the unstable platelet plug with chemically stable fibrin, follows and includes a series of interdependent, enzyme-mediated reactions. The endpoint of these reactions is the generation of thrombin, the enzyme that transforms the soluble protein fibrinogen to insoluble fibrin to stabilize the platelet plug.

The process of fibrin formation is normally well balanced and controlled, limiting it to the area of vessel injury. This localization prevents widespread or systemic coagulation activation. The procoagulant reactions are amplified to the appropriate degree, and natural inhibitors limit the proteolytic activity of the activated clotting factors. Negative feedback reactions also control fibrin formation: large amounts of thrombin destroy coagulation cofactors in the rate-limiting steps of its own production. The platelet–fibrin plug seals the injured vessel preventing blood loss, enabling the vessel to begin to repair itself, and allowing fibrin ultimately to be digested by plasmin, an enzyme of the fibrinolytic system.

Hemostasis is complex, involving many interrelating components and control mechanisms. Although no part of the system acts alone, each part is discussed individually, and then the concepts are integrated into a cohesive theory of hemostasis.



■ **FIGURE 32-1** The coagulation system. Activators and inhibitors of clotting and fibrinolysis keep the coagulation system in balance. Clotting occurs when blood vessels are damaged and activators of coagulation factors are exposed or released. Clotting is controlled because fibrinolysis is initiated in response to clotting activation. Inhibitors of both clotting and fibrinolysis serve to bring the system back into balance. An imbalance in the activation or inhibition of either clotting or fibrinolysis causes thrombosis or bleeding.

COAGULATION MECHANISM

The reactions involved in coagulation were initially described as occurring in a cascade[1] or waterfall-like[2] fashion in which circulating, inactive coagulation factor precursors, or **zymogens**, are sequentially activated to their enzyme forms. Originally, this process was thought to be initiated by two different pathways, as diagrammed in Figure 32-2 ■. We now know that this separation into two pathways does not reflect in vivo coagulation.[3] The **intrinsic pathway** requires enzymes and protein cofactors that are all present in plasma; the **extrinsic pathway** requires enzymes and protein cofactors present in plasma as well as an activator—tissue factor—not found in blood under normal conditions. Both converge in a third path called the **common pathway** to generate the fibrin clot. The traditional analysis of the coagulation process assigns each of the coagulation factors to one of these pathways as indicated in Table 32-1 ★.

Although the model of intrinsic, extrinsic, and common pathways does not represent in vivo coagulation, the concept of the three pathways is invaluable in understanding and interpreting the in vitro clotting assays used to evaluate coagulation. The activated partial thromboplastin time (APTT) measures the intrinsic and common pathways, whereas the prothrombin time (PT) measures the extrinsic and common pathways.

☑ CHECKPOINT 32-1

What is the major distinction between the so-called extrinsic and intrinsic pathways?

★ **TABLE 32-1** Coagulation Factors in Intrinsic, Extrinsic, and Common Pathways

Intrinsic Pathway	Extrinsic Pathway	Common Pathway
Prekallikrien (PK)	VII	X
HK	Tissue factor (TF; III)	V
XII		II
XI		I
IX		
VIII		

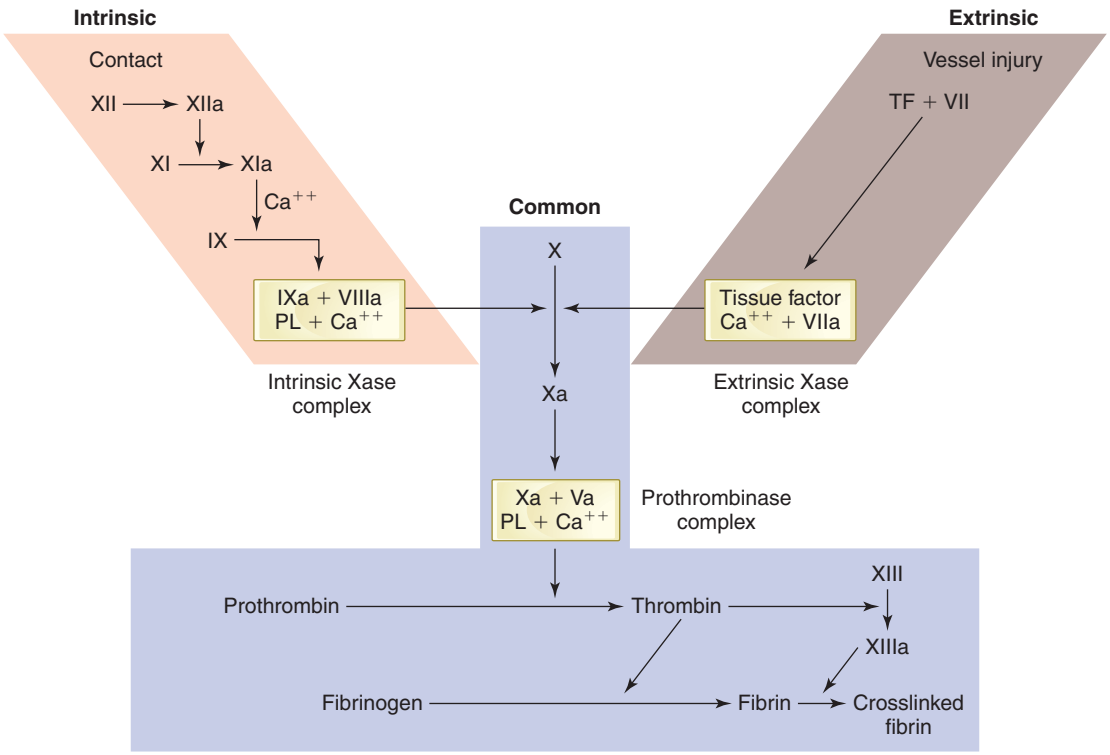


FIGURE 32-2 A simplified version of the coagulation cascade showing the cascade or waterfall-like sequence of reactions. The tan boxes indicate complex formation.

PL = platelet phospholipid; TF = tissue factor

CASE STUDY (continued from page 633)

Screening tests for evaluating hemostasis were done on Shawn. He was found to have a platelet count of 2.42×10^9/L. Bleeding time was 8 minutes. The PT was 29.5 seconds with a control of 12.0 seconds. The APTT was 51.0 seconds with a control of 55.4 seconds. Liver function tests were normal.

1. What do the results of the screening tests (platelet count, bleeding time, PT, and APTT) indicate?
2. What component of the hemostatic mechanism is most likely affected?

PROCOAGULANT FACTORS

Coagulation factors are the procoagulant proteins involved in hemostasis. The International Committee on Nomenclature of Blood Coagulation Factors[4] has assigned roman numerals I through XIII to some factors. Each roman numeral was assigned according to the order of discovery of the respective factor, not its place in the reaction sequence. Each factor has one or more common names or synonyms in addition to the roman numeral designation, although the common names are rarely used today (Table 32-2 ★). The letter *a* following the roman numeral indicates the activated form of the factor (i.e., it is no longer a zymogen but has active coagulant activity). There are, however, several exceptions to this terminology. In its activated form, factor II (prothrombin) is preferentially known as *thrombin* rather than FIIa. When fibrinogen (FI) is cleaved by thrombin, it is preferentially called *fibrin*. Tissue factor (TF) was assigned FIII, and calcium was assigned FIV, but these roman numeral designations are seldom used. FVI is no longer included in the coagulation sequence because it was originally assigned to a procoagulant activity subsequently found to be activated FV. High-molecular-weight kininogen (HK) and prekallikrein (PK) were never assigned roman numerals.

Most coagulation factors were discovered when physicians saw patients with a lifelong history of bleeding problems. Studies of the affected patients' blood revealed that certain proteins were functionally deficient. These proteins have been isolated and characterized as to their composition (including full amino acid sequences), biochemical functions, and the chromosomal location of their genes, providing useful information for understanding hereditary problems (Table 32-2).

CASE STUDY

3. What evidence indicates that Shawn has a hereditary bleeding disorder?
4. Are the nosebleeds significant in considering the diagnosis?

Properties of the Blood Coagulation Factors

Similarities among the structural and functional properties of the coagulation factors permit division into three groups: the **prothrombin group**, the **fibrinogen group**, and the **contact group** (Table 32-3 ★).

★ **TABLE 32-2** Summary of the Properties of Coagulation Factors

Factor	Common Name(s)	Circulating Form	$T_{1/2}$ (hours)	Role	Plasma Concentration	Chromosome (gene symbol)[a]
I	Fibrinogen	6-chain glycoprotein MW 340,000	72–120	Substrate; fibrin precursor	2000–4000 mcg (μg)/mL 10 nM	4 (*FGA, FGB, FGG*)
II	Prothrombin	Single-chain glycoprotein MW 72,000	60–70	Serine protease; thrombin precursor	100 mcg/mL 1390 nM	11 (*F2*)
III	Tissue factor	Single-chain, transmembrane glycoprotein MW 45,000	NA	Cofactor in extrinsic Xase; complex not found in circulation	NA	1 (*F3*)
IV	Calcium	Element	NA	Cofactor in some coagulation reactions	8.8–10.5 mg/dL	—
V	Proaccelerin	Single-chain glycoprotein MW 330,000	12–36	Cofactor in prothrombinase complex	10 mcg/mL 30 nM	1 (*F5*)
VII	Proconvertin, stable factor	Single-chain glycoprotein MW 50,000	3–6	Serine protease; constituent of extrinsic Xase complex	0.5 mcg/mL 10 nM	13 (*F8*)
VIII	Antihemophilic factor	Heterodimer glycoprotein MW 170–280,000	8–12	Complex with VWF in the circulation; cofactor in intrinsic Xase complex	0.15 mcg/mL ~1.0 nM	X (*F8*)
VWF	von Willebrand factor	Multimeric glycoprotein; subunit MW 250,000	12	Platelet adhesion; stabilization of circulating FVIII	10 mg/mL	12 (*vWF*)
IX	Plasma thromboplastin component	Single-chain glycoprotein MW 57,000	18–24	Serine protease; constituent of intrinsic Xase complex	5 mcg/mL 90 nM	X (*F9*)
X	Stuart factor	Two-chain glycoprotein MW 59,000	30–40	Serine protease; constituent of prothrombinase complex	5 mcg/mL 100 nM	13 (*F10*)
XI	Plasma thromboplastin antecedent	Two-chain glycoprotein MW 143,000	52	Serine protease; contact factor	4–6 mcg/mL 30 nM	4 (*F11*)
XII	Hageman factor	Single-chain glycoprotein MW 80,000	60	Serine protease; contact factor	23–39 mcg/mL 375 nM	5 (*F12*)
XIII	Fibrin-stabilizing factor	Heterotetramer glycoprotein MW 320,000	240	Transglutaminase; fibrin stabilizer	14–28 mcg/mL 65 nM	6 (A-chain)(*F13A1*); 1 (B-chain)(*F13B*)
HK	Fitzgerald factor, Williams factor, Flaujeac factor	Single-chain glycoprotein MW 120,000	156	Cofactor; complexed with PK and FXI; contact factor	70–90 mcg/mL 670 nM	3 (*KNG1*)
PK	Fletcher factor	Single-chain glycoprotein MW 88,000	35	Serine protease complexed with HK; contact factor	35–45 mcg/mL 410 nM	4 (*KLKB1*)

$T_{1/2}$ = biologic half-life; MW = molecular weight (in daltons); HMWK = high-molecular-weight kininogen; PK = prekallikrein

[a]Gene symbol approved by the HUGO Gene Nomenclature Committee

Prothrombin Group

The prothrombin group of procoagulant proteins includes prothrombin (FII) and factors VII, IX, and X. These factors have a molecular mass ranging from 50,000–100,000 daltons (Da). The factors in the prothrombin group are produced in the liver, and all contain the γ-carboxyglutamic acid-rich region called the *GLA-domain* that is critical for the calcium-binding properties of these proteins. The prothrombin group is also referred to as **vitamin K dependent** because its members require vitamin K to be functional (see the section "Vitamin K-Dependant Coagulation Proteins"). Other vitamin K-dependent proteins include protein C (PC), protein S (PS), and protein Z (PZ) (see the section "Biochemical Inhibitors").

★ **TABLE 32-3** Coagulation Factor Groups Based on Physical Characteristics

	Contact Group	Prothrombin Group	Fibrinogen Group
Characteristics	Requires contact with a negatively charged surface for activation	Requires vitamin K for synthesis; needs Ca^{++} to bind to a phospholipid surface	Large molecules; absent from serum (consumed in clotting)
Factors/Proteins included	XII, XI, PK, HK	II, VII, IX, X, PC, PS, PZ	I, V, VIII, XIII

PC = protein C; PS = protein S; PZ = protein Z

Fibrinogen Group

The fibrinogen group includes fibrinogen (FI) and FV, FVIII, and FXIII. Thrombin cleaves all four proteins during coagulation. These proteins have the highest molecular weights of all the factors ranging from 300,000–350,000 Da. They are not found in serum because they are consumed during clotting.

Contact Group

The contact group includes FXI and FXII, PK, and HK. These proteins have molecular weights ranging from 80,000–173,000 Da. The contact group is involved in the initial activation of the intrinsic pathway in vitro and requires contact with a negatively charged surface for activation. With the exception of FXI, the contact factors do not appear to play an essential role in hemostasis in vivo.[5] However, this group of factors is integrally related to other physiologic systems and provides a key link between inflammation and coagulation. In addition to coagulation, the activated contact factors can activate the fibrinolytic, kinin, and complement systems, playing an important role in the inflammatory response. These factors are not fully consumed during clotting and thus are found in serum (in reduced amounts).

Mechanism of Action of the Coagulation Factors

The coagulation cascade consists of a series of reactions in which limited proteolysis activates zymogens. Most of the newly generated proteases have low enzymatic activity until they bind to specific cofactors on appropriate phospholipid surfaces. Thus, the coagulation proteins can be grouped functionally as cofactors, substrates, or enzymes. The enzymes can be further grouped as either serine proteases or a transglutaminase.

Cofactors

FV and FVIII function as *cofactors* for activated coagulation proteases FXa and FIXa, respectively. The activated forms of these proteins, FVa and FVIIIa, have no enzymatic activity of their own. HK functions as a cofactor for the contact activation phase of coagulation (FXIIa and FXIa), PS is a cofactor for activated PC (see the section "Biochemical Inhibitors"), and TF is a cofactor for FVIIa. Each protease has some activity in the absence of its cofactor, but interaction with its cofactor significantly enhances proteolytic function.

Substrate

Fibrinogen is classified as a *substrate*: it is acted upon by the enzyme thrombin. It is the only coagulation protein that, as a substrate, does not participate in furthering the coagulation cascade.

Enzymes

The coagulation proteins that have enzymatic activity are secreted as zymogens, which are proenzymes or inactive precursors that must be modified to become active. Activation can involve either (1) a conformational change of the molecule or (2) proteolytic cleavage of one or more specific peptide bond(s). The coagulation zymogens are activated in a cascade-like sequence. Initially, a small number of zymogens are activated, and each sequentially activates the next zymogen in the cascade, resulting in the amplification of the initial stimulus.

Members of the family of **serine proteases**, including thrombin and factors VIIa, IXa, Xa, XIa, and XIIa, have a functional serine in their active sites and a common catalytic mechanism. They selectively cleave arginine or lysine peptide bonds in their substrates. Each serine protease involved in the coagulation cascade is highly specific for its substrate(s).

FXIIIa is the only coagulation enzyme with **transglutaminase** activity. It catalyzes the formation of peptide bonds between glutamine and lysine residues on fibrin, forming stable covalent cross-links.

Vitamin K-Dependent Coagulation Proteins

Seven of the blood coagulation proteins, including prothrombin, factors VII, IX, and X, and PC, PS, and PZ, require vitamin K for synthesis of the functional protein.

Function of Vitamin K

Vitamin K is a fat-soluble vitamin found in green leafy vegetables, fish, and liver. Some gram-negative intestinal bacteria also synthesize it. Vitamin K is necessary for the **γ-carboxylation** of glutamic acid residues clustered in a region of the proteins called the *GLA domain*. The addition of an extra carboxyl group (COOH) to the γ-carbon of the glutamic acid residues (γ-carboxylation) is a post-translational modification of the protein carried out by a specific γ-glutamyl carboxylase in the endoplasmic reticulum (ER) of the liver. The γ-carboxylation is essential for Ca^{++} binding, which induces a conformational change in the protein, and enables the factor to bind to negatively charged phospholipid surfaces (e.g., exposed phospholipids on activated platelets).[3] The carboxylase requires the reduced form of vitamin K (hydroquinone form) for its action. The carboxylation reaction converts reduced vitamin K to the epoxide form, which is converted back to the reduced form by the enzyme vitamin K epoxide reductase. Conversion back to the reduced form allows recycling of the vitamin K. Vitamin K antagonist drugs such as warfarin/Coumadin inhibit the activity of the vitamin K epoxide reductase and prevent recycling of vitamin K back to the reduced form. Warfarin/Coumadin overdoses can be reversed by vitamin K administration (Figure 32-3 ■).

Acarboxy Proteins

In the absence of vitamin K, the liver synthesizes the proteins, which can be found in the plasma. However, they are nonfunctional because they lack the γ-COOH groups necessary for Ca^{++}-phospholipid binding. The vitamin K-dependent factors lacking the COOH modification have previously been referred to as **PIVKA** (**p**rotein **i**nduced by **v**itamin **k** **a**bsence or antagonists) or des-γ-carboxy form. The International Committee on Thrombosis and Haemostasis recommends using the term *acarboxy* form. Warfarin/Coumadin, which inhibits the γ-carboxylation process, results in the production of inactive (acarboxy) proteins by the liver. The nonfunctional and functional forms of the factors are identical with respect to amino acid composition and antigenic determinants.

☑ CHECKPOINT 32-2

Will a vitamin K-deficient patient produce any of the vitamin K-dependent factors? Why is vitamin K so vital to the formation of coagulation complexes?

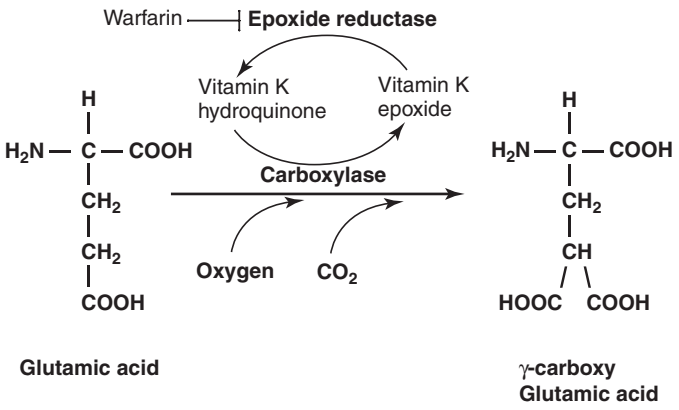


FIGURE 32-3 The vitamin K-dependent γ-carboxylation of glutamic acid. The coagulation factors in the prothrombin group must undergo this postribosomal carboxylation of glutamic acid residues in order to become functional. A specific carboxylase converts glutamyl residues to γ-carboxyglutamyl residues in a reaction requiring oxygen, carbon dioxide, and reduced vitamin K (hydroquinone). In the process, reduced vitamin K is converted to an epoxide and must be recycled to its reduced form by the enzyme epoxide reductase. This latter reaction is blocked by vitamin K antagonist drugs such as warfarin or Coumadin.

Structure of the Blood Coagulation Proteins

The blood coagulation proteins are made up of multiple functional units called *domains*, classified as catalytic or noncatalytic, depending on their function.[6] Catalytic domains contain the active site of the enzyme and are involved in activating other proteins. Noncatalytic domains contain regulatory elements. Many of the proteins involved in hemostasis contain similar structural domains and are believed to be derived from common ancestral genes (Web Figure 32-1).

Catalytic Domain

The *catalytic domain* of the serine proteases involved in blood clotting is highly homologous to trypsin. This domain's main function is to convert an inactive proenzyme to an active enzyme by cleaving a peptide bond in its target substrate, a process known as *zymogen activation by limited proteolysis*.

Noncatalytic Domains

The noncatalytic domains give each coagulation factor its own unique identity, making each highly specific in its activation and substrate activity. These domains are regulatory segments serving to bind calcium and promoting interaction with phospholipids, cofactors, receptors, and substrates. Some of the more common types of noncatalytic domains include the signal peptide, propeptide, γ-carboxyglutamic acid-rich GLA domain, epidermal growth factor domain, apple domain, finger domain, and kringle domain.

The *signal peptide* is a short domain that permits translocation of the protein to the endoplasmic reticulum for processing after synthesis. Vitamin K-dependent proteins have a *propeptide* between the signal peptide and the GLA domain, which contains the recognition site that directs γ-carboxylation of the vitamin K-dependent proteins after synthesis. The GLA domain contains 9–13 γ-carboxyglutamic acid residues (Gla) and is essential for Ca^{++} binding. When bound, Ca^{++} mediates the association of a coagulation factor with a phospholipid surface.

The *epidermal growth factor (EGF) domain*, *finger domain*, and *apple domains* are thought to be involved in binding to cofactors, activators, or substrates. The *kringle domain* is a lysine-binding site responsible for the affinity of certain proteins (plasminogen, plasmin, tissue plasminogen activator) for fibrin (Web Figure 32-2).

☑ CHECKPOINT 32-3

Why are the domains of the serine proteases involved in blood clotting so important in the hemostatic mechanism?

COAGULATION CASCADE

Complex Formation on Phosholipid Surfaces

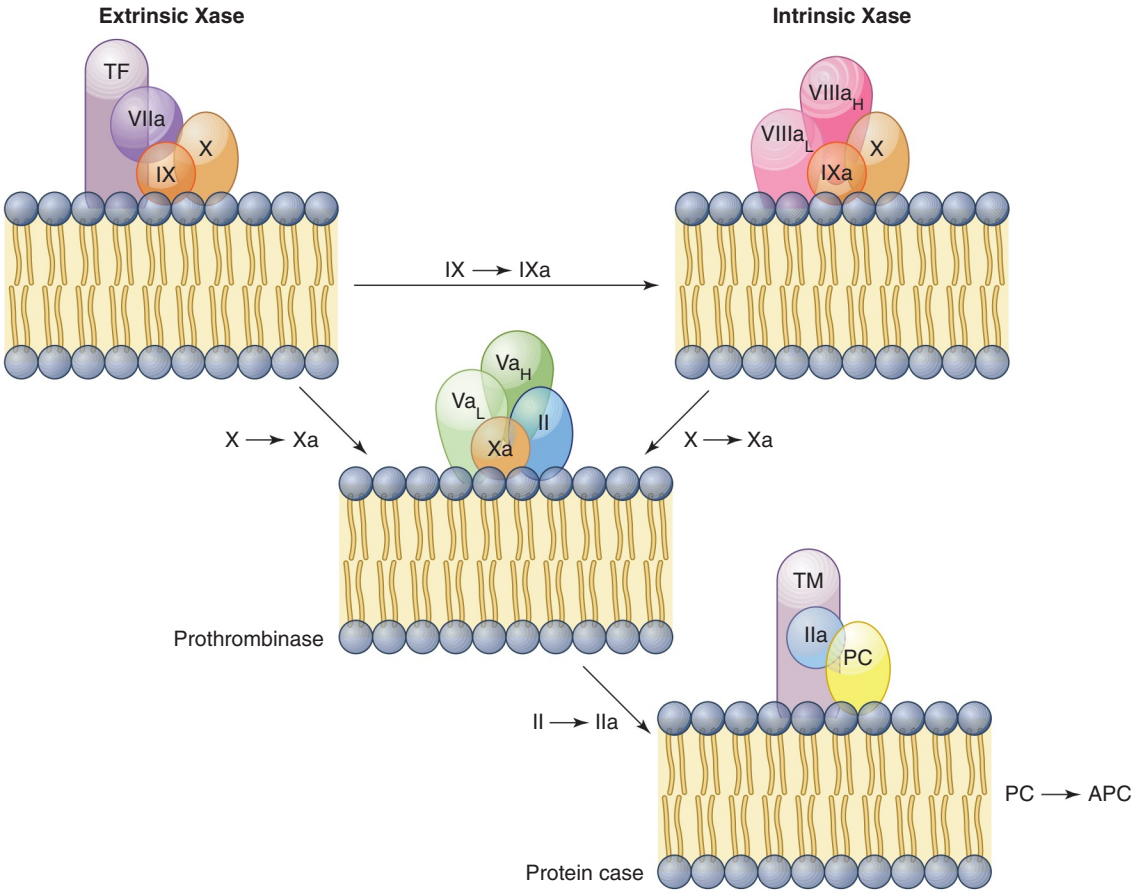
Most of the coagulation reactions occur on cell surface membranes. Clotting factors bind to the phospholipid membrane surface, forming a complex including enzyme, substrate, and cofactor.[7] Subendothelial tissue exposed when blood vessel injury occurs and the activated platelet surface provide the critical phospholipids for coagulation in vivo. The phospholipid surface serves to localize the reaction to the site of injury and increase the rate of activation by several orders of magnitude.[3] In addition, the reactions are generally protected from inhibitors in these cell surface–associated complexes.

Three procoagulant complexes, the **extrinsic Xase** (*extrinsic tenase*), **intrinsic Xase** (*intrinsic tenase*), and **prothrombinase complexes** assemble on the phospholipid membrane (Figure 32-4 ■). Extrinsic Xase is formed when TF, an integral membrane lipoprotein, is exposed to blood when vessel injury occurs. TF binds FVII or FVIIa in the presence of Ca^{++}, giving rise to the FVIIa/TF complex that activates FX. The intrinsic Xase is formed on membrane surfaces when FIXa and FVIIIa bind to phospholipid in the presence of Ca^{++}. This complex also activates FX to FXa. In a similar way, FXa and FVa bind to negatively charged membranes in the presence of Ca^{++} to form the prothrombinase complex. This complex converts prothrombin, also bound to the membrane, to thrombin. The rate of prothrombin activation by the prothrombinase complex is about 300,000 times faster than activation by FXa alone.[8]

The Intrinsic Pathway

Contact Factors/The Contact System

The originally described "intrinsic pathway" is more accurately called the *contact system* or *pathway*. The contact system complex also assembles on a surface. It is initiated when the four contact factors, FXII, FXI, PK, and HK, are activated when exposed to and adsorbed on negatively charged surfaces such as glass, kaolin, celite, and ellagic acid. Activation of these factors does not require Ca^{++}; thus, in vitro activation (or "preactivation") can occur in citrated patient plasma samples stored in glass tubes for prolonged periods before testing. Patients deficient in FXII, PK, and HK have no apparent clinical bleeding disorder despite a markedly prolonged APTT; thus, it is unlikely



■ **FIGURE 32-4** Schematic illustration of the coagulation complexes forming on a phospholipid surface. The vitamin K-dependent proteases (FVIIa, FIXa, FXa, and thrombin) are shown associated with their cofactors (tissue factor [TF], FVIIIa, FVa, and thrombomodulin [TM], respectively) and substrates (FIX, FX, prothrombin, and protein C [PC], respectively) on the membrane surface.

APC = activated protein C

that these factors play an important role in hemostasis in vivo. They do, however, contribute significantly to fibrinolysis, inflammation, complement activation, angiogenesis, and kinin formation. About 50% of patients with FXI deficiency have clinically evident bleeding abnormalities, suggesting that FXI could be an important accessory to blood coagulation, but it is probably not essential.

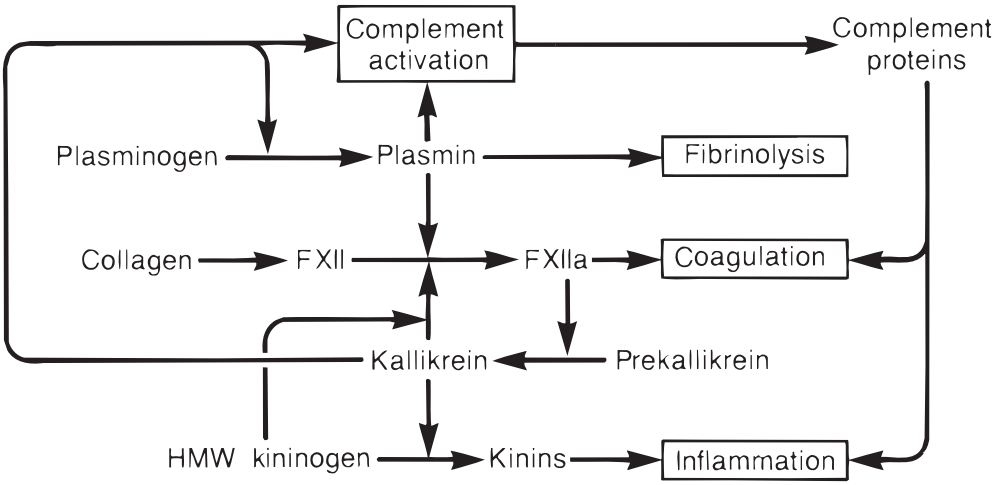
Interestingly, contact factors can activate ex vivo when blood comes in contact with nonbiologic surfaces, such as extracorporeal circulation systems used in cardiopulmonary bypass surgery. This ex vivo contact can result in a systemic inflammatory response in patients undergoing this type of surgery.[9] Attention recently has been refocused on the contact system. A number of studies now indicate that although it is not essential for hemostasis at sites of vessel injury, it does in fact contribute to pathologic thrombus formation[9] (Chapter 35).

Factor XII (FXII)

The liver produces FXII (Hageman factor). Zymogen single-chain FXII is proteolytically converted into the two-chain active serine protease FXIIa by kallikrein, plasmin (PLN), or "autoactivation." *Autoactivation* of FXII occurs by contact with negatively charged surfaces, which induce a conformational change that makes the protein more susceptible to autocatalysis. Many nonphysiologic substances (glass, kaolin, celite, ellagic acid)[10] have been associated with in vitro FXII autoactivation and are used as activators in the reagents for the APTT test. The nature of the physiologic surfaces responsible for autoactivation is still in question but is most likely negatively charged cellular membranes or subendothelial structures (collagen, basement membrane).

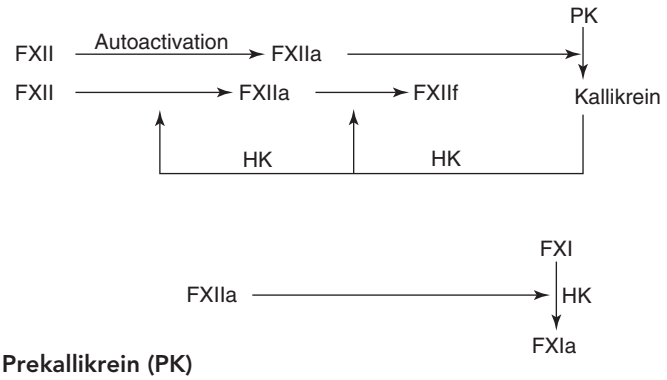
Bound FXIIa cleaves PK to kallikrein, which with HK as a cofactor can then reciprocally proteolytically activate surface-bound FXII to FXIIa (Web Figure 32-3). The generation of FXIIa and kallikrein by reciprocal activation amplifies these reactions. FXIIa can be further cleaved by kallikrein, yielding smaller FXII fragments (FXIIf) that have little or no procoagulant activity.[11]

In addition to activating PK to kallikrein, FXIIa has other enzymatic functions in hemostasis. As the first enzyme in the intrinsic pathway, FXIIa converts FXI to its active form, FXIa, in a reaction that requires HK as a cofactor. Second, FXIIa activates the fibrinolytic and complement systems. FXIIa, FXIa, and kallikrein can



■ **FIGURE 32-5** Role of contact factors in physiologic systems.

activate plasminogen directly, although much less efficiently than **tissue-type plasminogen activator (tPA)** or **urinary-type plasminogen activator (uPA)** (see the section "Physiologic Plasminogen Activators") (Figure 32-5 ■). FXIIa also activates the first component of the complement cascade (C-1).



Prekallikrein (PK)

PK predominantly circulates in plasma bound to HK (75% bound, 25% free).[11] FXIIa activates PK to plasma kallikrein. In addition to reciprocal activation of FXII and initiation of "intrinsic" coagulation, kallikrein can activate the kinin, fibrinolytic, and complement systems. Plasma kallikrein cleaves bradykinin (BK) from HK. PK directly activates plasminogen to plasmin and converts prourokinase (scuPA) to uPA, which in turn can activate plasminogen. Plasmin subsequently can activate the first and third components of the complement cascade (Figure 32-5). Plasma kallikrein also serves as a chemoattractant and activator for neutrophils and monocytes, facilitating the inflammatory response.

High-Molecular-Weight Kininogen (HK)

HK serves as a nonenzymatic cofactor in the activation of the contact factors, and is the source of BK. HK accelerates the rate of surface-dependent activation of FXII and of PK activation by FXIIa. HK binds to platelets, endothelial cells, and granulocytes, assembling the other components of the contact activation pathway. Two forms of kininogen, HK and low-molecular-weight kininogen (LK), are found in plasma. The liver produces both forms by alternative splicing of a single gene. HK, the preferred substrate for kallikrein, is a single-chain glycoprotein that can be cleaved by kallikrein into a two-chain molecule with the release of a small peptide, BK, a potent bioactive molecule. BK has many functions, including increasing vessel permeability, dilating small vessels, contracting smooth muscle, and causing pain. BK stimulates vasodilation, increasing blood flow, and stimulates endothelial cell profibrinolytic, antithrombotic, and antiplatelet properties by stimulating endothelial cell prostacyclin (PGI_2), nitric oxide (NO), and tPA synthesis and secretion (Chapter 31).

Factor XI (FXI)

In the contact pathway, FXI is activated to a serine protease by FXIIa and cofactor HK (Web Figure 32-4).

$$\text{FXI} \xrightarrow{\text{XIIa, HK}} \text{XIa}$$

Like PK, FXI circulates in the plasma primarily as a complex with HK. FXI is unique among the coagulation zymogens because it contains two identical polypeptide chains, each containing a catalytic site. Activation involves cleavage of an internal peptide bond in each of the polypeptide chains, resulting in a four-chain activated protease (FXIa). In addition to activation by FXIIa, both thrombin and FXIa itself can activate FXI, a positive feedback reaction. Thrombin is likely the physiologically relevant in vivo activator of FXI with minimal need for activation by FXIIa. Both FXI and thrombin bind to the surface of activated platelets where in vivo activation most likely occurs. The preferred substrate for FXIa is FIX, and its interaction with and activation of FIX likely also occurs on the surface of activated platelets.[12] Although patients with deficiencies of other contact factors (FXII, PK, HK) do not have bleeding problems, about 50% of patients with an FXI deficiency experience abnormal bleeding after surgery or injury. Plasma levels of FXI are not the only determinant of whether bleeding occurs because patients who have bleeding problems and those who do not can have similar plasma concentrations of FXI.[12]

Other Factors in the Intrinsic Pathway

Factor IX (FIX)

FIX is a single-chain vitamin K-dependent zymogen containing 12 Gla residues. In the intrinsic pathway, FXIa activates FIX in the presence of Ca^{++} and does not require any other cofactor (Web Figure 32-5). Activation is associated with cleavage of two bonds in FIX, releasing an activation peptide and a two-chain molecule, FIXa.

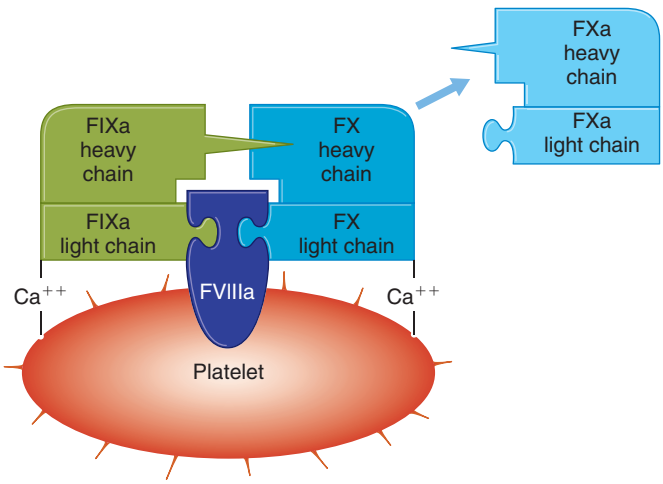
$$\text{IX} \xrightarrow[Ca^{++}]{\text{XIa}} \text{IXa}$$

When activated, FIXa forms a complex (intrinsic Xase) with cofactor FVIIIa and Ca^{++} on the surface of activated platelets to activate FX (Figure 32-6 ■).

$$\text{X} \xrightarrow[Ca^{++}]{\text{IXa + VIIIa, activated platelet surface}} \text{Xa}$$

The extrinsic pathway complex FVIIa/TF also activates FIX by cleavage at the same amino acids in zymogen FIX (see the section "The Extrinsic Pathway"). This extrinsic pathway for FIX activation bypasses the contact activation system.

FIXa (but not FIX) binds to phospholipid surfaces on activated platelets in the presence of calcium via its GLA domain; it does not bind to resting platelets. It is one of two coagulation proteins (FVIII and FIX) whose genes are encoded on the X chromosome. The severe bleeding that results from a deficiency of FIX (Hemophilia B) indicates that it plays a critical role in blood coagulation.



■ **FIGURE 32-6** The complex formed by the sequential activation of the intrinsic pathway (FIXa, FVIIIa, Ca^{++}) activates FX. The cofactor, FVIIIa, binds to the platelet phospholipids and orients FIXa and FX to enhance activation of FX. FIXa and FX are bound to the platelet surface via Ca^{++} bridges. Spikes on platelet indicate activated platelet.

Factor VIII (FVIII)

FVIII is synthesized primarily in the liver by sinusoidal endothelial cells and circulates in plasma in association with von Willebrand factor (VWF) (Figure 34-1). Each protein in this complex is under separate genetic control and has distinct biologic functions and immunologic properties (Chapter 34). Binding to VWF stabilizes FVIII and protects it from inhibition or degradation. FVIII circulates in the plasma in an inactive form until it is activated by thrombin (or FXa), yielding FVIIIa, which serves as a cofactor for the FIXa activation of FX.

FVIII is synthesized as a large precursor protein consisting of the linear array of A1:A2:B:A3:C1:C2 domains (Figure 32-7 ■). The A domains are involved in protein–protein interactions, the B domain is removed during activation, and the C domains are involved in binding to phospholipid surfaces.[13] A signal peptide is cleaved on translocation into the lumen of the ER. FVIII is further processed on secretion from the cell with cleavage at two sites within the B domain, releasing part of the B domain and a heterodimer composed of a heavy chain (A1:A2 and the remaining part of the B domain) and a light chain (A3:C1:C2 domains). In the plasma, FVIII associates with VWF, which stabilizes FVIII and prolongs its circulating half-life from ~2 hours to ~12 hours.[14] In addition to increasing FVIII survival, VWF also is involved in regulating FVIII activity. VWF binds to FVIII, preventing FVIII from binding to phospholipids and activated platelets and protecting it against inactivation by activated protein C.[15] It does not, however, prevent thrombin activation of FVIII but facilitates thrombin proteolytic cleavage.[13] Thrombin cleaves both the heavy and the light chains of FVIII, creating a three-polypeptide molecule (FVIIIa) with subunits derived from the A1, A2, and A3:C1:C2 domains and releasing the residual B domain.[13] The A1 and A3:C1:C2 subunits remain linked as a stable dimer while the A2 subunit, associated primarily by weak electrostatic interactions, readily dissociates. As a result, FVIII is labile, and its activity is rapidly lost at room temperature. Thrombin activation of FVIIIa dissociates FVIIIa from the protective influence of VWF, permitting FVIIIa interaction with the activated platelet surface via its C domains.[13] FVIIIa has no enzymatic activity but as part of the intrinsic Xase complex functions as a cofactor for FIXa activation of FX, increasing the catalytic activation of FX ~10^6-fold. However, large amounts of FXa and thrombin destroy the procoagulant function of FVIIIa. Like FIX, the gene for FVIII is located on the X chromosome, and FVIII (also called *antihemophilic factor*) is critical for normal blood coagulation. A deficiency of FVIII results in Hemophilia A.

von Willebrand Factor (VWF)

VWF is a large multimeric glycoprotein synthesized by endothelial cells and megakaryocytes (Figure 34-1). VWF is synthesized as a pre-pro-VWF molecule with a signal peptide, a large propeptide (VWF propeptide [VWFpp]), and the mature VWF protein. VWF undergoes extensive intracellular modifications during trafficking through the ER and Golgi, including removal of the signal peptide, formation of C-terminal–linked dimers, and subsequently higher N-terminal–linked multimers, glycosylation, and protein folding.[16]

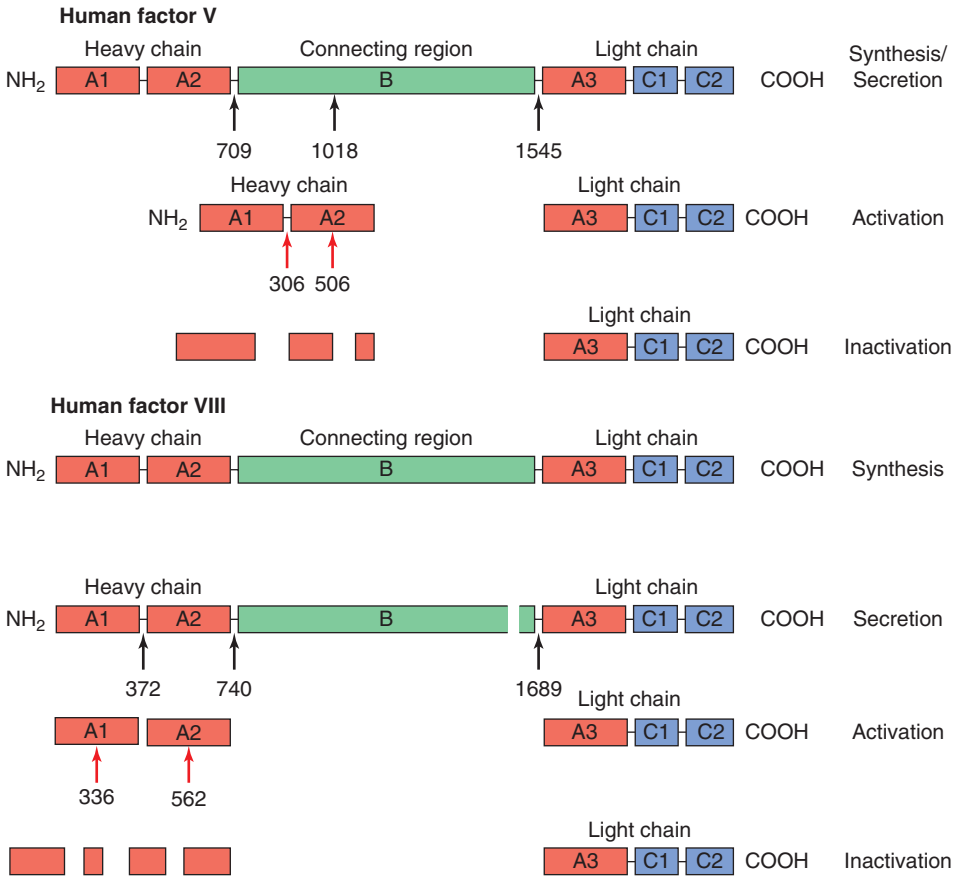


FIGURE 32-7 Structural domains in FV and FVIII. Black arrows indicate thrombin cleavage sites red arrows indicate protein C cleavage sites for inactivation. The letters A, B, and C indicate the identity of the domains.

The propeptide is cleaved from pro-VWF by the enzyme furin, releasing the VWFpp and the mature VWF monomer.[17] The VWF monomer consists of a structure with A, B, C, and D domains (Figure 34-2). The VWFpp is cleaved during processing, and appears to play a role in multimerization and the intracellular storage of VWF because both the cleaved VWFpp and VWF multimers are stored in the secretory granules.[16] VWF is synthesized as a monomer of 250,000 Da but circulates in the plasma as a family of molecules (multimers) of a wide range of sizes (0.5 to >20 million Da). The largest forms are most effective in promoting platelet adhesion; however, all multimers can bind FVIII in a 1:1 molar ratio.

Mature VWF multimers are stored in secretory granules, α-granules (αGs) of platelets and Weibel-Palade bodies of endothelial cells. They are released from these intracellular stores following injury or stimulation by thrombin, ADP, epinephrine (platelets), and histamine or the vasopressin derivative 1-deamino-8-D-arginine vasopressin (DDAVP) (endothelial cells). Endothelial cells can secrete VWF from the luminal surface into the plasma and from the abluminal surface into the subendothelial cell matrix. Secretion from intracellular stores releases both VWF and VWFpp. Plasma levels of VWFpp can be measured and help to differentiate some subtypes of von Willebrand disease (Chapter 34).

Platelet and endothelial cell VWF generally include larger multimers than are found in plasma, called *ultralarge VWF multimers* (*ULVWF*). Degradation of ULVWF to the normal sizes found in plasma is accomplished by the VWF-cleaving protease, **ADAMTS-13**, a member of the **a** **d**isintegrin-like **a**nd **m**etalloprotease with **t**hrombo**s**pondin repeats proteins.[18] The ULVWF are released from the Weible-Palade bodies and remain tethered to the endothelial cell surface, forming long stringlike structures. When tethered, ULVWF is exposed to the shear forces of the flowing blood, and the protein unfolds and exposes the cleavage site for ADAMTS-13.[19] If the ULVWF is released directly into plasma, platelets can spontaneously aggregate, resulting in thrombosis (Chapter 35).

VWF contributes to both primary and secondary hemostasis. It mediates adhesion of platelets to the vessel wall in areas of high flow rate and high shear force by simultaneously binding VWF to GPIb/IX on platelet surfaces and to collagen and elastin in the subendothelium (Chapter 31). High-molecular-weight multimers of VWF contain the highest number of platelet and other cell surface–binding sites. VWF also can bind to the glycoprotein IIb/IIIa (GPIIb/IIIa) receptor on platelets (the normal physiologic receptor for fibrinogen) and thus can promote platelet aggregation when plasma fibrinogen levels are low. These molecules serve as *intercellular bridges* between platelets,

between platelets and subendothelium, and between platelets and endothelial cells. In secondary hemostasis, VWF carries FVIII in the plasma, binding to and stabilizing FVIII via a noncovalent interaction between the two molecules. The two proteins circulate as a VWF/FVIII complex. It has been suggested that VWF in the circulating complex binds to platelet GPIb, thus delivering FVIII to platelets adhering to damaged endothelium. After proteolytic activation of FVIII, FVIIIa is released from VWF and interacts with the platelet surface in assembling the Xase complex.[13]

☑ CHECKPOINT 32-4

Which components of the intrinsic pathway are believed to be essential for in vivo hemostasis?

The Extrinsic Pathway

FX can also be activated by the extrinsic pathway involving FVIIa and its cofactor, tissue factor. When vessel injury occurs, nonvascular cells (fibroblasts, vascular adventitial cells) with TF on their surface are exposed to the blood. TF binds FVII and FVIIa in the presence of Ca^{++}, forming the FVIIa/TF complex. Once formed, this complex initiates the extrinsic pathway of blood coagulation, converting FX to FXa (extrinsic Xase complex). The FVIIa/TF complex also can activate FIX of the intrinsic pathway, bypassing the need for contact activation of this pathway. FXa, thrombin, and FVIIa itself can feed back to activate more FVII to FVIIa.

$$\begin{array}{ll} \text{VIIa/TF} & \text{VIIa/TF} \\ \downarrow Ca^{++} & \downarrow Ca^{++} \\ \text{X} \rightarrow \text{Xa} & \text{IX} \rightarrow \text{IXa} \end{array}$$

Tissue Factor (TF)

TF is the cellular receptor and cofactor for FVII and FVIIa. It is a transmembrane glycoprotein originally referred to as *FIII*, or *tissue thromboplastin*. TF is expressed constitutively on the plasma membrane of most nonvascular cells. Cells that are in contact with flowing blood do not normally express it, but monocytes and endothelial cells can be stimulated to produce TF by endotoxin, complement component 5a, immune complexes, interleukin-1, tumor necrosis factor, and thrombin.[15] TF and the platelet phospholipid surface have similar functions in coagulation; both attract calcium ions to facilitate the formation of procoagulant enzyme complexes at the injury site.

Factor VII (FVII)

FVII is one of the vitamin K-dependent proteins produced by the liver and has 10 Gla residues. FVII circulates in two forms: the inactive zymogen, FVII, and low levels of the active enzyme, FVIIa (~1% of the total plasma FVII).[20] Free FVIIa has little if any activity in the absence of TF. However, the trace levels of circulating FVIIa are sufficient to initiate the coagulation cascade when injury exposes TF. FVII is a single-chain molecule activated to a two-chain FVIIa by proteolytic cleavage of a single peptide bond with the two peptides remaining tethered by a disulfide bond. No cleavage peptide is released.

As with intrinsic activation of coagulation, there is positive feedback in activating the extrinsic pathway. When TF and FVII are bound in a complex, FVIIa can autocatalyze more FVII to FVIIa. In addition, FXa and FIXa associated with a phospholipid surface can feed back to activate FVII, further increasing the amount of FVIIa formed.[21]

☑ CHECKPOINT 32-5

Historically, major importance for initiating coagulation was assigned to either the intrinsic or extrinsic pathway. What are some observations that suggest that the classic concepts were not accurate?

The Common Pathway

The intrinsic and extrinsic pathways converge on the common pathway as both pathways activate FX.

Factor X Activation

FX can be activated by both the FVIIa/TF/Ca^{++} complex (extrinsic Xase) or the FIXa/FVIIIa/Ca^{++}/phospholipid complex (intrinsic Xase).

$$\begin{array}{c} \text{VIIa/TF/Ca}^{++} \\ \downarrow \\ \text{X} \rightarrow \text{Xa} \\ \uparrow \\ \text{IXa/VIIIa} \\ \text{Ca}^{++}\text{/PL} \end{array}$$

Thrombin Generation

FXa then forms a complex with cofactor FVa, phospholipid, and Ca^{++}. This prothrombinase complex acts to optimally activate prothrombin to thrombin.

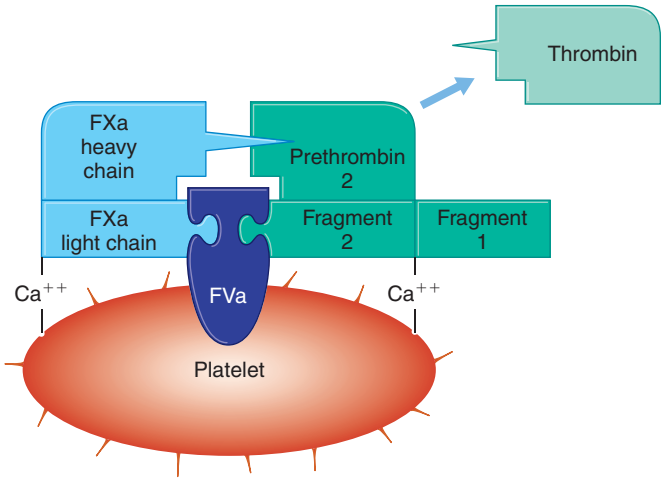
$$\begin{array}{c} \text{Xa/Va/Ca}^{++}\text{/PL} \\ \downarrow \\ \text{Prothrombin} \rightarrow \text{Thrombin} \end{array}$$

Factor X (FX)

FX is another vitamin K-dependent protein produced by the liver; it contains 11 Gla residues. It circulates as a two-chain disulfide-linked zymogen. FVIIa/TF and FIXa/FVIIIa release a 52-amino acid activation peptide from the FX heavy chain to generate FXa. Russell's viper venom (RVV) protease can also directly activate FX.

Factor V (FV)

FV is a large glycoprotein primarily produced by the liver. It is also called a *labile factor* because, like FVIIIa, its activity deteriorates quickly at room temperature. About 20% of FV in the blood is found in the αGs of platelets internalized from the plasma by endocytosis.[13,22] FV circulates in the plasma as a single-chain molecule with a domain organization similar to FVIII (A1:A2:B:A3:C1:C2 domains)



■ **FIGURE 32-8** The prothrombinase complex (FXa, FVa, Ca^{++}) activates prothrombin to thrombin. FVa binds to the platelet phospholipid surface and orients FXa and prothrombin to enhance the formation of thrombin. FXa and prothrombin are bound to the platelet surface via Ca^{++} bridges. This complex is very similar to the complex formed by the intrinsic pathway (FIXa, FVIIIa, Ca^{++}, PL). Spikes on platelet indicate activated platelet.

(Figure 32-7). FV and FVIII share a high degree of amino acid identity (~40% over the A1,A2,A3,C1,C2 domains).[13] Activation of FV to FVa with full cofactor activity requires cleavage of three peptide bonds. Both thrombin and FXa can activate FV, although thrombin is the primary activator in vivo. Cleavage produces a two-chain heterodimer consisting of a heavy chain (A1:A2 domains) noncovalently linked through Ca^{++} to a light chain (A3:C1:C2 domains). The B domain is released as a result of activation. FVa binds to phosphatidylserine on the activated platelet surface via the C domains and serves as the attachment site for FXa via the A2 domain (Figure 32-8 ■). Most of the FVa that participates in the prothrombinase complex on the platelet membranes probably is the result of secretion of FV from activated platelet αGs. FVa increases the catalytic activation of prothrombin ~1000× by properly orienting the FXa and prothrombin molecules on the phospholipid surface.[13]

Prothrombin (FII)

Prothrombin is a vitamin K-dependent protein produced by the liver and contains 10 Gla residues. Zymogen FII circulates as a single-chain molecule and is bound to phospholipid surfaces via its GLA domain and Ca^{++}. The prothrombinase complex cleaves prothrombin in two places. The first cleavage generates a two-chain disulfide-linked protease called *meizothrombin*; the second cleavage releases an activation peptide (fragment 1.2), which contains the GLA domain, producing the active enzyme thrombin. The alternative sequence of cleavage, first releasing the C-terminal half of the molecule that contains the catalytic domain (single-chain prethrombin) and the prothrombin fragment 1.2 followed by prethrombin cleavage to thrombin plays a minor role in vivo.[23] Assays for fragment 1.2 reflect the level of in vivo prothrombin activation.

Thrombin is composed of an α- and β-chain linked by a single disulfide bond. It does not contain the GLA domain of prothrombin and thus does not bind to negatively charged phospholipids (Figure 32-9 ■). As a result, thrombin does not remain tethered to the phospholipid surface of activated platelets on which it was formed but can dissociate and move to other areas or surfaces (e.g., thrombomodulin [TM] on nearby intact endothelial cells).

Roles of Thrombin

Thrombin generation is critical for normal hemostasis; it has a number of diverse roles, both procoagulant and anticoagulant (Figure 32-10 ■). Thrombin functions as a procoagulant by cleaving fibrinopeptides A and B from fibrinogen to create fibrin monomer; it also generates other procoagulant activities, activating factors Va, VIIIa, XIa, and XIIIa to greatly amplify its own formation. Thrombin stimulates endothelial cells to release VWF and plasminogen activator inhibitor (PAI-1) and to express TF[24]. Thrombin also is a potent activator of platelets, stimulating platelet shape change, secretion and aggregation, and acidic phospholipid exposure on the outer membrane. Thrombin can suppress fibrinolysis by activating the **thrombin-activatable fibrinolysis inhibitor (TAFI)**. Conversely, thrombin also has antithrombotic functions that dampen its own formation. Thrombin binds to TM, and activates protein C, and stimulates endothelial cells to release tPA, prostacyclin, and endothelium-derived relaxing factor (EDRF, also known as nitric oxide [NO]). In addition to its role in hemostasis, thrombin has mitogen and cytokine-like activities and plays a role in inflammation, wound healing, angiogenesis, and atherothrombosis.[23]

CASE STUDY (continued from page 635)

5. What coagulation factors are included in the extrinsic pathway?
6. What factors are included in the intrinsic pathway?
7. What factors are included in the common pathway?
8. An abnormal PT and a normal APTT would indicate a problem with which factor?

Formation of Fibrin

The insoluble fibrin clot is formed from soluble fibrinogen in three distinct steps:

1. *Proteolytic cleavage* of arginine–glycine bonds in fibrinogen by thrombin, releasing fibrinopeptides A and B from the α- and β-chains, forming fibrin monomer
2. *Spontaneous polymerization* of fibrin monomers to form fibrin polymers
3. *Stabilization* of the fibrin polymers by FXIIIa-catalyzed cross-linking

Fibrinogen

The fibrinogen molecule is a large (340,000 Da), trinodular glycoprotein composed of three pairs of polypeptide chains found in plasma and platelet αGs. Megakaryocytes and platelets do not

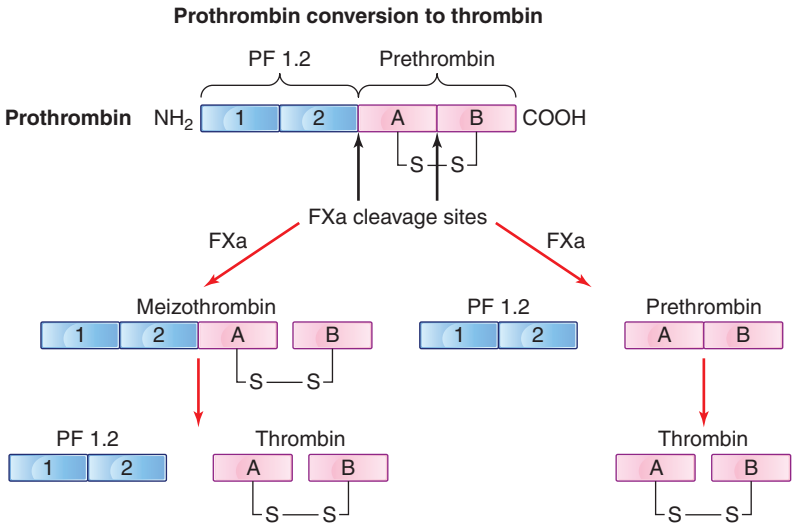


FIGURE 32-9 Pathways for prothrombin cleavage. The prothrombin molecule is a single-chain glycoprotein that can be divided into the "pro" portion (prothrombin fragment [PF] 1.2) and the thrombin portion. Activation of prothrombin requires two cleavages by FXa, which proteolytically cleaves the molecule between the "pro" and thrombin portions, releasing PF 1.2 and prethrombin. Prethrombin is then further cleaved into two disulfide bonded chains forming the potent enzyme thrombin. Alternatively, FXa can cleave the latter bond first, releasing a two-chain protease called *meizothrombin*, followed by the cleavage that releases fragment 1.2, producing the active enzyme thrombin. The latter pathway appears to be the predominant pathway in vivo.

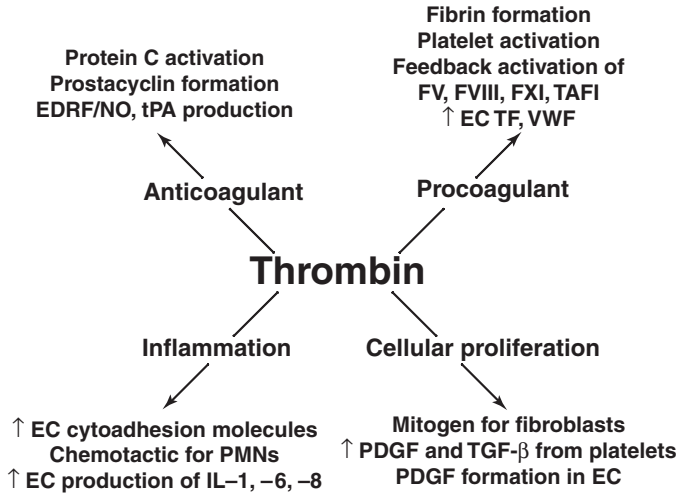


FIGURE 32-10 The multifactorial roles of thrombin. In addition to its multiple roles as a procoagulant protease, thrombin also has anticoagulant functions and important activities in promoting inflammation and cellular proliferation.

EDRF = endothelium-derived relaxing factor; NO = nitric oxide; tPA = tissue plasminogen activator; TAFI = thrombin-activatable fibrinolysis inhibitor; EC = endothelial cell; TF = tissue factor; VWF = von Willebrand factor; PMNs = polymorphonuclear neutrophils; PDGF = platelet-derived growth factor

synthesize the protein but absorb it from plasma. Fibrinogen is the most abundant coagulation protein with a plasma concentration of 2–4 mg/mL (representing about 2% of the total plasma protein concentration). The three different polypeptides of fibrinogen (Aα, Bβ, γ) are encoded by three separate genes located on chromosome 4, and the production of the three chains is coordinately regulated. Fibrinogen is also one of several hepatic proteins whose production is increased as part of the acute phase response mediated by IL-6.

Fibrinogen exists as a dimeric protein, consisting of three pairs of nonidentical polypeptide chains; thus, a molecule of fibrinogen would be described as $A\alpha_2$, $B\beta_2$, γ_2[25] (Figure 32-11 ■). This nomenclature reflects the fact that small polypeptides (fibrinopeptides A and B [FPA, FPB]) are released from Aα- and Bβ-chains by thrombin, producing a single molecule of fibrin, α_2, β_2, γ_2. Twenty-nine disulfide bonds join the three pairs of chains. Electron microscopy has demonstrated the folding of the molecule into a trinodular structure.[26] The central nodule, the E region, is referred to as the *N-terminal disulfide knot* (*N-DSK*) because the amino terminal ends of all six polypeptide chains join to form this region. The E region contains the fibrinopeptides A and B. The two outer nodules, the D regions, are made up of the carboxy-terminal ends of the β- and γ-chains and a short sequence of the α-chain. The Aα-chain has a long polar region at the carboxy-terminal end that folds back toward

the N-DSK, which is the site of initial plasmin hydrolysis (see the section "Fibrinogen Degradation Products"). The D and E regions are separated from each other by 111–112 amino acids forming a triple-stranded rodlike α-helical structure called the *coiled-coil region* (supercoiled region).

Release of Fibrinopeptides (FPs) A and B

Thrombin binds to the central E region of fibrinogen and cleaves specific arginine–glycine bonds, releasing four peptides from the fibrinogen molecule. A short 16-amino acid peptide is released from each Aα-chain (FPA) as well as a 14-amino acid peptide from each Bβ-chain (FPB). The resulting molecule is called a *fibrin monomer*. Release of FPA alone is sufficient for the fibrin assembly process.

Assembly of Fibrin Polymers

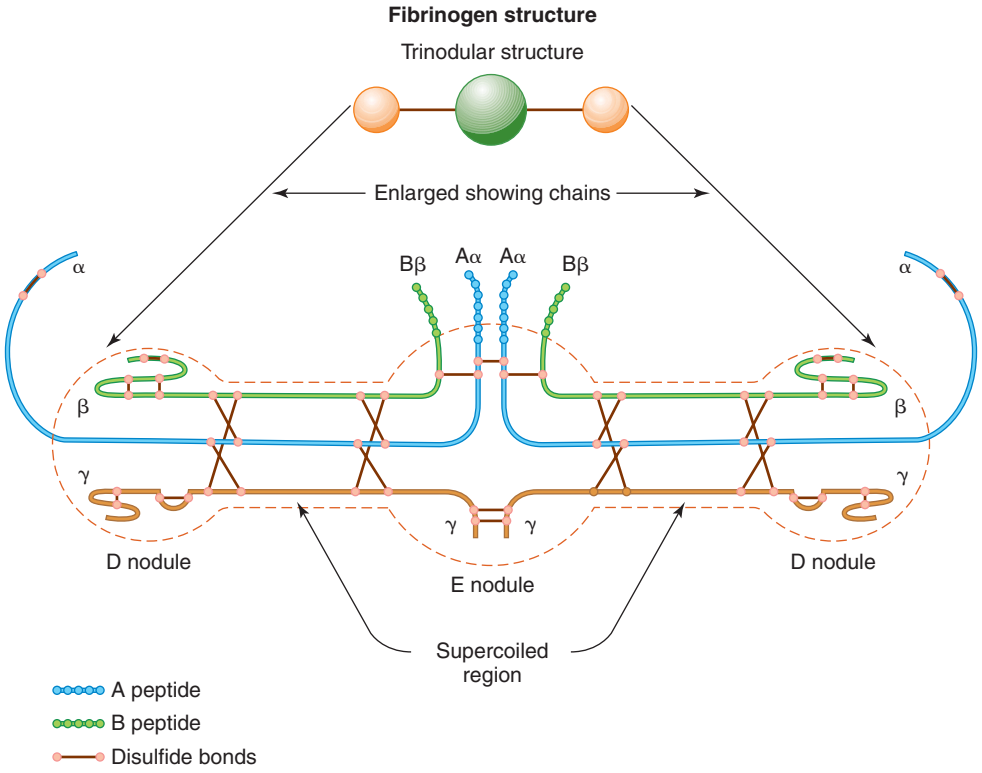
The cleavage of FPA and FPB exposes binding sites in the central E nodule that interact with complementary sites on the γ-chain of the D nodule of other fibrin monomers, creating a D–E contact. The polymerization sites on the D nodule are always available and interact with the complementary sites on the E nodule exposed after thrombin cleavage of FPA and FPB. The spontaneous polymerization continues by the addition of a third monomer forming a D–D contact as well as another D–E contact. The use of x-ray crystallography to study fibrinogen has provided insights into the mechanism by which fibrin self-assembles.[27] The nodules' noncovalent interaction leads to the initial formation of two-stranded polymers termed *protofibrils*. The polymer strands aggregate in an overlapping pattern called *half-staggered array* in which the second strand is offset from the first by half of the length of a single fibrin molecule. The protofibrils then aggregate into thick fibers through lateral associations held together by weak noncovalent and electrostatic interactions. During fibrin monomer polymerization, other plasma proteins including components of the fibrinolytic system (e.g., plasminogen, PAs, fibrinolytic inhibitors) also bind to the fibrin surface. Thrombus extension in vivo can be blunted by unactivated fibrinogen molecules or fibrin degradation products (FDPs) that can bind to and cap the ends of a protofibril.

Fibrin Stabilization

Spontaneously polymerized fibrin strands are unstable, but the α- and γ-chains of adjacent fibrin strands are subsequently covalently cross-linked by FXIIIa. The cross-linking strengthens the clot and provides resistance to chemical (5M urea) and enzymatic (plasmin) digestion compared with the uncross-linked polymer (Figure 32-12 ■).

Factor XIII (FXIII)

The final reaction in fibrin formation is the stabilization of the fibrin polymer catalyzed by FXIIIa. FXIII circulates as a heterotetramer (A_2B_2), which is the product of two separate genes coding for the



■ **FIGURE 32-11** Formation of fibrin polymer. Fibrinogen is a trinodular structure composed of three pairs (Aα, Bβ, γ) of disulfide-bonded polypeptide chains. The central nodule is known as the *E region*. Thrombin cleaves small peptides, A and B, from the α- and β-chains in this region to form fibrin. The central nodule is joined by supercoiled α-helices to the terminal nodules also known as the *D regions*.

A- and B-chains. FXIII is found evenly distributed between plasma and platelets. Platelet FXIII exists as an A_2 dimer lacking the B subunit. The plasma FXIII A chain is primarily synthesized by bone marrow cells (megakaryocytes, monocytes, macrophages) and is stored in the soluble (nongranular) fraction of the platelet. The A-chain contains the active site of the enzyme, the fibrin binding, and substrate recognition domains. The B-chains are secreted by hepatocytes and complex rapidly with the A-chains in the plasma, stabilizing the hydrophobic A_2 subunit in the plasma.[28] The heterotetramer circulates in association with fibrinogen.

When fibrin is polymerized, plasma FXIII is bound as the A_2B_2 complex. Fibrin binding provides a mechanism to localize FXIIIa activation to sites of thrombin generation and fibrin polymerization, allowing for efficient activation. Thrombin is not able to activate FXIII without the presence of fibrin, which acts as a cofactor. Thrombin cleavage of the A-chains, dissociating the B-chains and releasing an activation peptide, activates FXIII to FXIIIa. The expression of FXIIIa activity requires its interaction with calcium ions, which induce a conformational change, exposing its active center and promoting catalysis.

FXIIIa is thus a calcium-dependent transglutaminase that catalyzes the formation of covalent peptide bonds within the fibrin polymer. These bonds are formed between the terminal D-regions of γ-chains of two adjacent fibrin monomers within a protofibril, forming longitudinal end-to-end cross-links. More slowly, cross-links form between polar appendages of neighboring monomers' α-chains, promoting lateral aggregation of fibrils. The covalently cross-linked fibrin network produces a fibrin clot with increased mechanical strength and increased resistance to proteolytic digestion by plasmin. The presence of these unique bonds is responsible for the liberation of specific FDPs (D-dimers; see the section "Fibrin Degradation") when plasmin digests the clot. In contrast to the hydrogen-bonded polymers, the FXIIIa–stabilized fibrin polymer is not soluble in 5M urea or monochloroacetic acid (Chapter 36). In addition to forming covalent cross-links between molecules of fibrin, FXIIIa also cross-links fibrinolytic inhibitors to the fibrin clot, including α_2-antiplasmin, plasminogen activator inhibitor-2 (PAI-2), and TAFI.[28] The localization of these fibrinolytic inhibitors to the fibrin clot results in the inhibition of plasmin formation and protects the clot from premature lysis. Finally, FXIIIa cross-links fibrin to extracellular matrix molecules, helping to anchor the fibrin clot to the vessel wall and promoting wound healing.

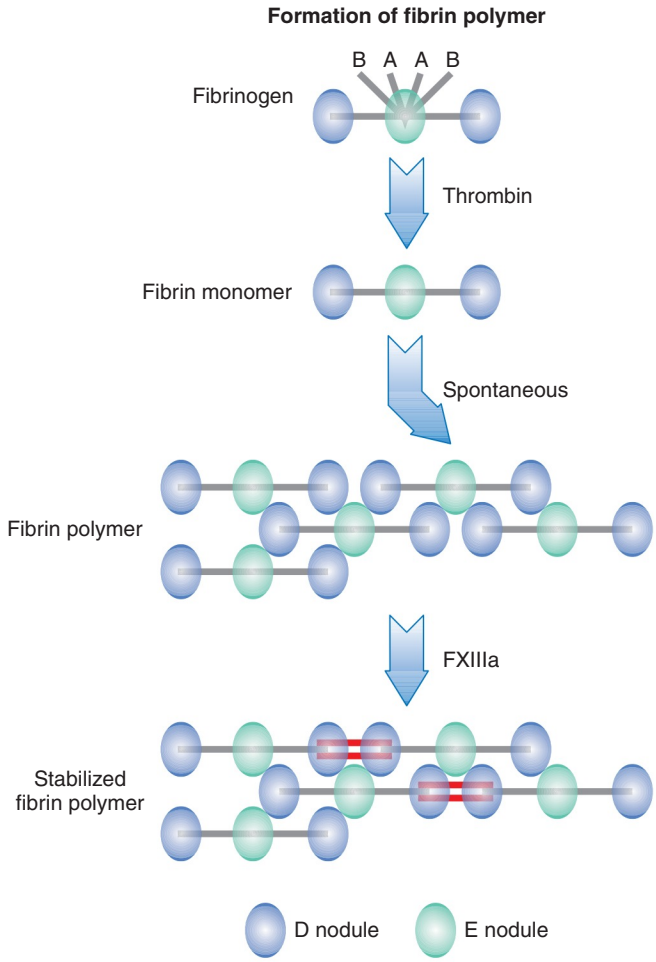


FIGURE 32-12 Formation of fibrin polymer. Thrombin cleaves the A and B fibrinopeptides from the E nodule of fibrinogen to form fibrin monomer. The cleavage permits the spontaneous growth of fibrin polymers as the E nodule assembles with the D nodules of other fibrin monomers. Hydrogen bonds initially join the polymer. FXIIIa in the presence of Ca^{++} is responsible for catalyzing the formation of covalent bonds between glutamine and lysine residues of adjacent monomers (D nodules), thus stabilizing the lattice formation.

☑ CHECKPOINT 32-6

What are the three steps in the formation of an insoluble fibrin clot?

FIBRINOLYTIC SYSTEM

Hemostasis requires not only the formation of a fibrin clot to stop bleeding but also the lysis of the clot following repair to the vessel wall, restoring normal blood flow through the vessel. The process of removing fibrin is called **fibrinolysis**. The fibrinolytic system is activated in response to activation of the coagulation cascade. This system functions to remove fibrin from the vascular system in a controlled manner once it has fulfilled its hemostatic function; it also serves to prevent excessive fibrin accumulation. This system digests the fibrin clot through proteolysis, and must be regulated so that proteolytic activity is limited to the area of fibrin formation and does not occur prior to healing of the vascular lesion. Excessive local or systemic fibrinolysis can result in bleeding; an inadequate fibrinolytic response can cause delayed lysis of a thrombus and contribute to excess fibrin accumulation.

Introduction

Fibrin formation occurs both intravascularly and extravascularly as a consequence of coagulation, inflammation, and tissue repair. When no longer needed, fibrin must be removed so that normal

vessel and tissue structure and function can be restored. The fibrinolytic system is responsible for dissolving thrombi and maintaining a patent vascular system. The key components in this system are (1) the inactive proenzyme, plasminogen (PLG), (2) plasminogen activators (PAs), (3) the active enzyme plasmin (PLN), (4) fibrin, (5) fibrin/fibrinogen degradation products (FDPs), and (6) PA inhibitors (PAIs) (Table 32-4 ★).

Like the coagulation system, the fibrinolytic system normally acts locally at sites of fibrin accumulation without causing systemic effects. If the fibrinolytic system's activity increases (e.g., deficiencies of inhibitors α_2-antiplasmin or PAI-1), the hemostatic plug's stability can be compromised or premature lysis can result in an increased bleeding tendency. Conversely, decreased activity of the system (e.g., deficiency of plasminogen, PAs, or elevated inhibitors) can result in delayed or inadequate fibrin dissolution.

Fibrin formation essentially initiates fibrinolysis by assembling the various fibrinolytic components to optimize and localize their interactions. When clotting begins, PLG binds to fibrin throughout the developing thrombus. tPA also binds to fibrin, increasing its enzymatic activity so it can efficiently convert PLG to PLN. The formation of a ternary complex between the activator (tPA), the zymogen (PLG), and the substrate (fibrin) results in the targeted, specific degradation of fibrin (not circulating fibrinogen or other plasma proteins). PLN digests fibrin to soluble degradation products, producing several well-characterized fibrin fragments. The system is inhibited by the action of PAIs and by the PLN inhibitor, α_2-antiplasmin (AP) (also known as α_2-plasmin inhibitor [PI]).[29]

When associated with the fibrin surface, PLN is protected from rapid inhibition by α_2-AP so that efficient degradation of a fibrin clot can occur (Figure 32-13 ■). If free PLN leaks into the circulation, these PLN molecules are rapidly inactivated by α_2-AP. Thus, the activity of this potentially dangerous proteolytic enzyme is localized to the fibrin clot. The relative proportions and locations of profibrinolytic (PLG and PA) molecules and the antifibrinolytic molecules (PAIs and α_2-AP) influence the timing and degree of clot dissolution.

☑ CHECKPOINT 32-7

Why is the process of fibrinolysis a vital part of the hemostatic mechanism, and why must it be closely regulated and controlled?

Plasminogen (PLG) and Plasmin (PLN)

PLG is a single-chain glycoprotein synthesized by the liver. It has five kringle domains that are critical in regulating fibrinolysis. The kringles contain lysine-binding sites (LBSs) that are responsible for the binding affinity of PLG and PLN for lysine residues on fibrin, cell surface receptors, and other proteins including α_2-AP. PLG preferentially binds to C-terminal lysines as opposed to intramolecular lysines. **Plasminogen activators (PAs)** convert PLG into its active two-chain form, the serine protease PLN, by hydrolysis of a single peptide bond. PLN exhibits much broader substrate activity than the procoagulant serine proteases and has the potential to degrade and destroy most proteins. In addition to fibrin, PLN can degrade essentially all proteins susceptible to trypsin including fibrinogen, FV and FVIII, complement, several hormones, and the glycoprotein receptors on the surface of platelets. However, PLN formation and inactivation are highly regulated in vivo, and the enzyme is active only temporarily and locally (in the vicinity of the fibrin clot). PLG binds to fibrin, and activation occurs much more efficiently at the fibrin surface, targeting the proteolytic action of the enzyme PLN to its substrate.

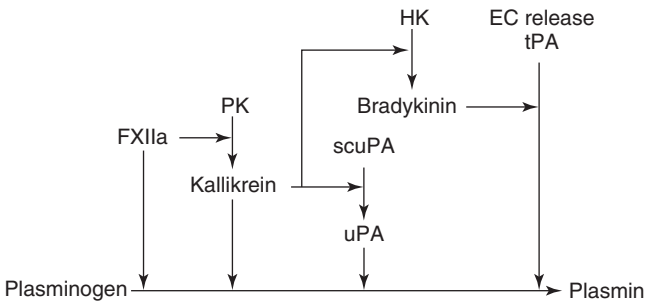
The active enzyme PLN has a positive feedback effect on fibrinolysis. Intact plasma PLG has an amino terminal glutamic acid residue (Glu-PLG). PLN can cleave Glu-PLG, producing a smaller PLG molecule with a lysine N-terminal amino acid (Lys-PLG). Lys-PLG has a higher affinity for binding to fibrin and greater reactivity with PAs, thus accelerating and improving the efficiency of PLN formation.[29]

Activators of Fibrinolysis

The two major PAs that occur in the circulating blood are tPA and uPA, also called urokinase (UK). The contact phase of coagulation produces serine proteases (FXIIa and kallikrein), which also can promote PLG activation.

Contact Plasminogen Activator (PA) Pathway

PAs are generated during the contact phase of the intrinsic coagulation pathway. FXIIa, FXIa, and kallikrein all have the ability to directly activate PLG to PLN, although somewhat slowly. However, the relative plasma concentrations of FXII compared with tPA and uPA suggest that it might be an effective PA in vivo.[29] Contact pathway PAs normally account for ~15% of total PLN-generating activity in plasma.[30] Kallikrein can affect fibrinolysis by converting single-chain uPA (scuPA) to its more active two-chain form, tcuPA (usually represented by just uPA) and by liberating bradykinin from HK. Bradykinin can then stimulate release of tPA from endothelial cells, further enhancing fibrinolysis.



Physiologic Plasminogen (PLG) Activators

The two important physiologic plasma PAs are tPA and uPA, also known as UK, named for their tissue source of original purification. Both are serine proteases that have a high degree of specificity for converting PLG to PLN by the cleavage of a single bond (Arg561-Val). Each has different structural, functional, and immunologic properties.

★ **TABLE 32-4** Summary of Properties of the Components of the Fibrinolytic System

Component	Description	Role	$T_{1/2}$	Concentration	Chromosome (gene symbol)[a]
I. Fibrinolytic component					
Plasminogen (PLG)	Single-chain glycoprotein MW 92,000	Zymogen; precursor of plasmin	2.2 days	200 mcg/mL 2000 nM	6 (*PLG*)
Plasmin (PLN)	Dimeric glycoprotein MW 92,000	Serine protease; cleaves fibrin	0.1 sec	—	—
II. Activators of plasminogen					
Tissue plasminogen activator (tPA)	Glycoprotein MW 68,000	Serine protease; complexed with inhibitor PAI-1; plasminogen activator	4 min	0.005 mcg/mL 0.07 nM	8 (*PLAT*)
Urokinase type plasminogen activator (uPA)	(scuPA) MW 54,000	Serine protease; low enzymatic activity until cleaved to tcuPA by PLN; plasminogen activator	7 min	0.002 mcg/mL 0.04 nM	10 (*PLAU*)
Factor XII	Single-chain glycoprotein MW 80,000	Serine protease	2–3 days	30 mcg/mL 375 nM	5 (*F-12*)
Prekallikrein	Single-chain glycoprotein MW 88,000	Serine protease	7–10 days	40 mcg/mL 450 nM	4 (*KLKB1*)
III. Inhibitors					
Plasminogen activator Inhibitor-1 (PAI-1)	Single-chain glycoprotein MW 52,000	Proteinase inhibitor (Serpin): tPA and uPA inhibitor	8 min	0.001–0.02 mcg/mL 0.1–0.4 nM	7 (*SERPINE1*)
Plasminogen activator inhibitor-2 (PAI-2)	Glycoprotein—(two forms, MW 60,000 [secreted], MW 47,000 [intracellular])	Proteinase inhibitor (Serpin): tPA, uPA inhibitor	—	<0.005 mcg/mL </0.07 nM 0.250 mcg/mL in late pregnancy	18 (*SERPINE2*)
α_2-antiplasmin (AP)	Single-chain glycoprotein MW 70,000	PLN inhibitor; also kallikrein, thrombin, tPA inhibitor	3 days	70 mcg/mL 1000 nM	17 (*PLI*)
Thrombin activated fibrinolysis inhibitor (TAFI)	Single-chain protein MW 60,000	Carboxypeptidase B: plasminogen activation inhibitor by cleaving lysine AA from fibrin	10 min	5 mcg/mL 75 nM	13 (*CPB2*)
C-1 esterase inhibitor (C-INH)	Single-chain glycoprotein MW 105,000	C1r and C1s, FXIIa, FIXa, kallikrein, plasmin inhibitor	70 hr	180 mcg/mL 1700 nM	11 (*SERPING1*) 12 (*A2M*)
α2-Macroglobulin (α_2-M)	Glycoprotein: 4 identical chains MW 725,000	Inhibitor of multiple proteases by "trapping"		2500 mcg/mL 3000 nM	
IV. Cell surface receptors					
uPA Receptor (uPAR)	Glycoprotein receptor MW 55,000	uPA receptor on surface of EC, monocytes, and macrophages	—	—	19 (*PLAUR*)
Annexin 2	Heterotetramer MW 38,000	tPA, PLG receptor on EC, monocytes, and macrophages, SMC	—	—	15 (*ANXA2*)
Low-density-lipoprotein Receptor-like protein (LRP)	MW 600,000	Clearance receptor for uPA/PAI-1 and PAI-2, tPA/PAI-1, PLN/α_2PI	—	—	12 (*LPR1*)

MW = molecular weight in Da; Serpin = serine protease inhibitor; SMC = smooth muscle cells

[a]Gene symbol approved by the HUGO Gene Nomenclature Committee

Tissue-Type Plasminogen Activator (tPA)

Vascular endothelial cells of small vessels mainly produce tPA, which seems to be the predominant PA activity in the circulatory system. In vivo, tPA is constitutively released from endothelial cells as a single-chain molecule (sctPA); a secondary pool is released from storage vesicles by stimuli such as thrombin, bradykinin, histamine, exercise, venous stasis, shear stress, and DDAVP administration.[29] tPA has two kringle domains containing lysine-binding sites (LBSs) that are involved in fibrin binding, fibrin-specific PLG activation, rapid clearance in vivo, and binding to endothelial receptors. sctPA is unique among the serine proteases because it does not circulate as a zymogen but is fully active toward its substrate PLG in its single-chain form.

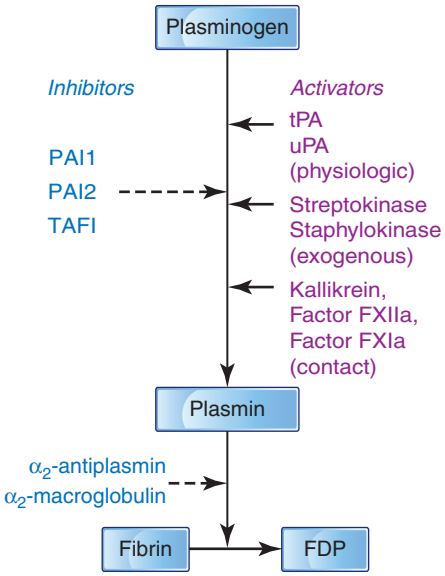


FIGURE 32-13 The fibrinolytic system. This system can be activated by the physiologic activators, tissue plasminogen activator (tPA) derived from endothelial cells, and urinary-type plasminogen activator (uPA) or the contact activators, kallikrein, FXIIa, and FXIa. Exogenous activators, streptokinase and staphylokinase, can also activate the system. Plasmin, the product of activation, breaks down the fibrin clot by digesting fibrin to fibrin degradation products (FDPs). The inhibitors of plasminogen activation, plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2), thrombin-activatable fibrinolysis inhibitor (TAFI), and the inhibitors of plasmin (α_2-antiplasmin and α_2-macroglobulin) control the fibrinolytic system. The fibrinolytic system, like the clotting system, is intricately regulated by these activators and inhibitors.

However, PLG activation by sctPA in plasma (in the absence of fibrin) is inefficient. The activation of PLG by sctPA is markedly increased when both proteins are bound to fibrin via their respective LBSs. sctPA can be converted to a two-chain molecule (tctPA) by cleavage of a single peptide bond by kallikrein, PLN, or FXa. However, the single-chain form is nearly as effective as the two-chain form in the presence of fibrin.[31]

In addition to binding to and functioning on a fibrin surface, tPA also associates with the membrane of endothelial cells. The endothelial cell receptor for tPA is **annexin II (A2)**, which functions as a coreceptor for both tPA and PLG. Binding and activation of tPA and PLG help maintain a fibrinolytic potential on undamaged vascular surfaces.

Urinary-Type Plasminogen Activator (uPA)

uPA is primarily produced by renal tubular epithelium, monocytes, macrophages, and vascular endothelium and is found in urine and plasma. However, uPA functions mainly in the tissues where it plays an important role in digesting the extracellular matrix, enabling cells to migrate.[32] This process is important in wound healing, embryogenesis, inflammation, and cancer metastasis. uPA is secreted as a scuPA but has little proteolytic activity until it is converted to a two-chain form by PLN, FXIIa, or kallikrein.[33] Unlike tPA, uPA does not require fibrin as a cofactor and can activate both fibrin-bound and circulating PLG. The localized generation of small amounts of fibrin-bound PLN converts scuPA to tcuPA (usually represented by just uPA), bringing about the reciprocal activation of PLG and scuPA.

A specific receptor for uPA, **uPA receptor (uPAR)**, plays an important role in localizing uPA-catalyzed PLG activation. The receptor binds scuPA and uPA to the endothelial cell surface and facilitates activation of scuPA to uPA and of PLG to PLN. Binding uPA to uPAR increases the activation of plasminogen 20-fold by colocalization of uPA and PLG and contributes to a fibrinolytic potential on undamaged endothelium.[29]

Lipoprotein(a) (Lp[a]), a low-density lipoprotein (LDL)-like particle similar in structure to PLG, has been shown to inhibit the activation of PLG by tPA and uPA.[34] Lp(a), which also possesses LBS, competes with PLG and tPA for fibrin binding, interfering with PLG activation. Thus, elevated levels of Lp(a) are antifibrinolytic and are considered a thrombotic risk factor (Chapter 35).

Exogenous Activators

In addition to these physiologically important endogenous PLG activators, several bacterial species also produce efficient PLG activators.[29] Streptokinase (SK), derived from β-hemolytic streptococci, is not a serine protease but acts by forming a 1:1 complex with PLG. When bound to SK, PLG undergoes a conformational change, exposing an altered active site that can then bind and cleave a second molecule of PLG to form PLN. SK is used as a therapeutic agent to dissolve clots but is highly antigenic, and formation of antibodies often limits its clinical usefulness. SK has no preferential action toward PLG bound to fibrin and thus acts equally well on PLG in the circulation, resulting in extensive systemic plasmin activation and a generalized proteolytic state. PAs are also produced by *Staphylococcus aureus* (staphylokinase [SAK]) and by *Yersinia pestis*. Like SK, SAK is not an enzyme but forms a 1:1 complex with PLG. However, SAK does not activate PLG in the absence of fibrin. Trace amounts of PLN can convert the SAK/PLG complex to a SAK/PLN that is a highly fibrin-specific PA.

Fibrin Degradation

PLN is responsible for the asymmetric, progressive degradation of fibrin (or fibrinogen), forming distinct protein fragments referred to as **fibrin degradation products (FDPs)**. The liver rapidly clears these fragments from the circulation. Their detection in the plasma is of diagnostic value for some hemostatic disorders.

Fibrinogen Degradation Products (FDPs)

The sites of PLN proteolytic action are similar in fibrinogen and cross-linked fibrin. PLN digestion of fibrinogen has been widely studied and has been used as the model to explain the digestion of fibrin. The products formed include fragments X, Y, D, and E (Figure 32-14 ■). Fragment X is formed when plasmin cleaves a few small peptides from the exposed polar appendages in the C-terminal region of the α-chains of fibrinogen and amino acids β1–42 from the N-terminal portion of the β-chains. Next asymmetric cleavage in one coiled-coil region midway between one D terminal nodule and the E central nodule produces a uninodular fragment D and a binodular fragment D-E (fragment Y). PLN then cleaves fragment Y in the coiled-coil region between the

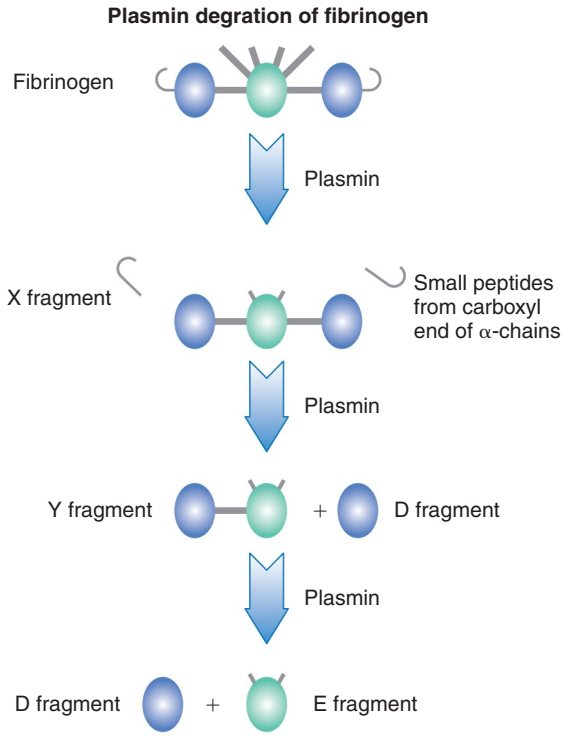


FIGURE 32-14 Plasmin degradation of fibrinogen. The degradation of fibrinogen by plasmin occurs in well-defined sequential steps. First, small peptides are cleaved from the carboxyl ends of α-chains producing fragment X. The E nodule retains the A and B peptides. The fragment X is still capable of reacting with thrombin to form fibrin. Next one of the D nodules is cleaved from the fragment X, producing fragment Y (DE) and a fragment D. Additional cleavage of the fragment Y produces D and E fragments.

remaining E and D nodules, producing free fragment E and another fragment D. Each fibrinogen molecule ultimately is degraded into two D fragments, one E fragment, and a few small peptides.

Fibrin Degradation Products (FDPs)

PLN degradation of fibrin monomer and non-cross-linked fibrin polymer is essentially identical to that of fibrinogen degradation. PLN degradation of cross-linked fibrin, however, is slower and produces unique degradation products because of the intermolecular bonds induced by FXIIIa. The smallest unique degradation product, the D-D fragment, consists of two fragment D segments of two adjacent cross-linked fibrin monomers. To completely separate adjacent D and E nodules on neighboring molecules, six cleavages must occur (2α-, 2β-, and 2γ-chains cleaved). Digestion severing all connections is not always complete. Therefore, some of the cross-linked D-D degradation products are found within molecules of various molecular weights. They can consist of various combinations of X, Y, D, and E fragment complexes from two or more cross-linked fibrin monomers (e.g., DD/E, YD/DY, YY/DXD) (Figure 32-15 ■). The D-dimer test, a test for fibrin breakdown products, is a specific marker for PLN degradation of fibrin (Chapter 36).

If present in sufficient concentration, the fibrin fragments can exert an anticoagulant effect on the clotting system. Fragment X can still bind and be cleaved by thrombin but very slowly. Fragment X can compete with fibrinogen for thrombin, so less fibrin is formed. Fragments Y, D, and E can inhibit the polymerization of fibrin by competing for polymerization sites on fibrin monomers. The fragments can also interfere with primary hemostasis by inhibiting platelet aggregation.

Systemic Effects of Plasmin

If free in plasma, PLN can cause proteolytic degradation of numerous plasma proteins, including fibrinogen; factors V, VIII, and XII; and components of the kinin and complement systems. The rapid formation of complexes between free PLN and its inhibitor, α_2-AP (P-AP), controls this potentially dangerous proteolytic process. If PLN inactivation is not sufficiently controlled, the result is a systemic fibrinolytic state characterized by PLG activation, depletion of α_2-AP and

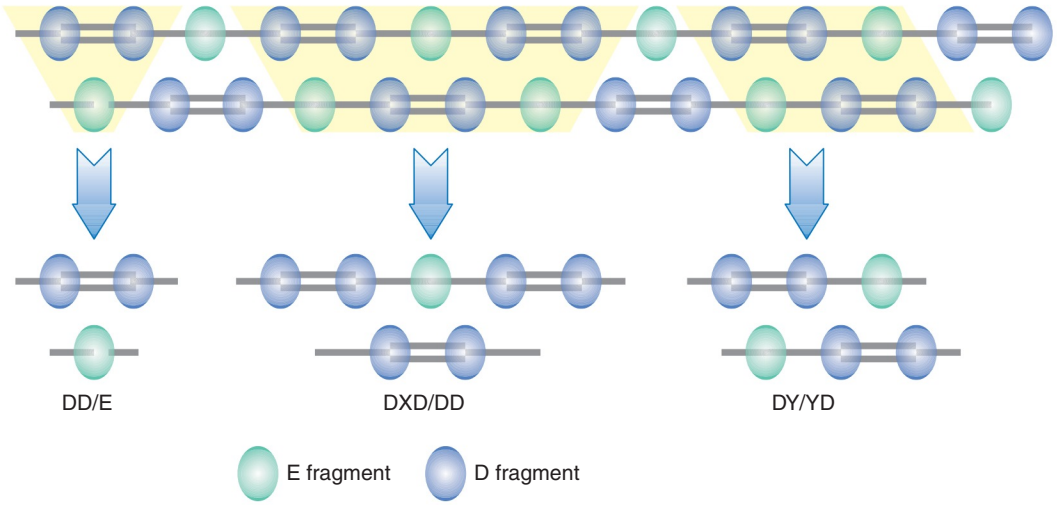


FIGURE 32-15 Plasmin digestion of fibrin occurs at cleavage sites identical to those of fibrinogen. However, because of the covalent bonds of the FXIIIa-stabilized polymer, digestion is slower. Derivatives also are different; some sites are not accessible in the lattice formation. This schematic drawing depicts some of the derivatives of plasmin digestion of fibrin.

fibrinogen breakdown. Physiologic fibrinolysis, which occurs when PLN is attached to fibrin, is highly fibrin specific and not associated with a systemic fibrinolytic/proteolytic state.

☑ CHECKPOINT 32-8

How are the PLN degradation products of fibrinogen and fibrin different?

Inhibitors of Fibrinolysis

Fibrinolytic inhibitors, which modulate the fibrinolytic system to prevent systemic proteolysis, hold fibrinolytic proteins in check (Table 32-4). They act at the PLG activation step or directly on PLN.

Plasminogen Activator Inhibitors (PAIs)

Several inhibitors control the activation of PLG. Most belong to a family of serine-protease inhibitors called **serpins** that inhibit target molecules by forming a 1:1 stoichiometric complex. The primary regulators are the PAIs. Five proteins with PAI activity have been identified: PAI-1, PAI-2, PAI-3, protease nexin-1, and neuroserpin. Of these, only PAI-1 appears to play a significant role in regulating PA activity in the vasculature and most tissues.[29] The interaction of PAI-1 with tPA is >1000 times greater than the interaction of tPA with the other PAIs.

Plasminogen Activator Inhibitor-1 (PAI-1)

Endothelial cells, monocytes, macrophages, megakaryocytes, hepatocytes, and adipocytes produce PAI-1, which appears to be the primary physiological inhibitor of tPA and uPA. PAI-1 reacts with tPA, sctPA, and uPA but not scuPA. In plasma, PAI-1 is in significant excess over tPA (Table 32-4), and most of the tPA circulates in a complex with PAI-1. Release of PAI-1 from activated platelets into the developing hemostatic plug ensures that the initial fibrin matrix is not prematurely lysed by tPA activation of PLG. PAI-1 is an acute-phase reactant protein. Thrombin, IL-1, TGF-β, TNFα, and endotoxin induce dramatic increases in plasma PAI-1 levels. Elevated PAI-1 results in a decrease in tPA activation of PLG and a shift in the hemostatic balance toward hypercoagulability (Chapter 35). Deficiency of PAI-1 results in a serious bleeding disorder because of unregulated and excessive fibrinolysis.

Plasminogen Activator Inhibitor-2 (PAI-2)

PAI-2, the second plasminogen activator inhibitor, is found in highest amounts in placental tissue and macrophages. Plasma levels are very low except in pregnancy when they are drastically elevated. PAI-2 inhibits both tPA and uPA but is less effective toward sctPA.

Activated Protein C Inhibitor (PAI-3)

The protein originally identified as PAI-3 is now thought to be the activated protein C (APC) inhibitor.

Lipoprotein Receptor-Related Protein (LRP)

PA/PAI complexes are removed from the circulation by binding to the low-density lipoprotein receptor-related protein (LRP) receptors on hepatocytes and macrophages. LRPs mediate clearance of free tPA, tPA/PAI-1, uPA/PAI-1, tPA- or uPA-PAI-2, and α_2-macroglobulin/protease complexes.[29]

Thrombin-Activatable Fibrinolysis Inhibitor (TAFI)

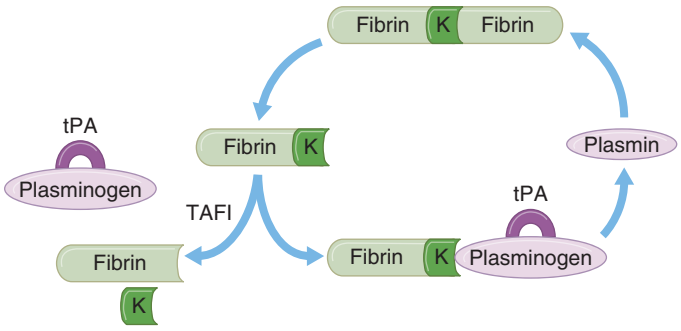
TAFI is a plasma protein zymogen (procarboxypeptidase B) produced by the liver that inhibits fibrinolysis when activated to TAFIa by the thrombin/thrombomodulin complex.[35] Activation requires the formation of a ternary complex involving TAFI-thrombin-TM. PLG binding to fibrin occurs primarily at carboxy-terminal lysine residues. New C-terminal lysines are generated during initial plasmin degradation of fibrin. TAFIa suppresses fibrinolysis by removing C-terminal lysine residues from fibrin, thereby eliminating the plasminogen-binding sites on fibrin[36] (Figure 32-16 ■). TAFIa thus protects the fibrin clot from degradation by inhibiting the binding and activation of PLG. Because of TAFIa's short half-life (~10 minutes), this effect is temporary, delaying fibrinolysis activation until the fibrin clot can be established and stabilized. Thus, sufficient thrombin generation initially results in the suppression of fibrinolysis by activating TAFI, which in turn plays a major role in the balance between fibrin deposition and removal. TM also has a role in downregulating coagulation by activating protein C (see the section "Biochemical Inhibitors").

α_2-Antiplasmin (AP)

The liver produces and secretes α_2-AP, which is also found in platelet αG. α_2-AP is the principal physiologic inhibitor of PLN; it binds via LBS on PLN and rapidly inhibits the enzyme. In the circulation, α_2-AP reacts quickly with free PLN, but PLN bound to fibrin is protected from inactivation because fibrin occupies the same binding sites on PLN as would AP.[29] Circulating α_2-AP thus makes an important contribution to limiting systemic fibrinolysis. α_2-AP can also bind PLG, interfering with its absorption to fibrin. Some α_2-AP molecules are cross-linked to fibrin by FXIIIa during clotting, thus increasing the initial resistance of fibrin to the action of PLN, and allowing repair processes to start before clot lysis is initiated.

α_2-Macroglobulin (α_2-M)

α_2-macroglobulin (α_2-M) is a backup inhibitor for several proteases, including PLN and the other hemostatic serine proteases. If the fibrinolytic system has been extensively activated and a large amount of PLN has been generated, α_2-AP's inhibitory capacity would be exhausted. Then



■ **FIGURE 32-16** Downregulation of plasmin formation by TAFI. Fibrinolysis is initiated when plasminogen is converted to plasmin by tPA. Both plasmin and tPA preferentially bind to carboxy-terminal lysine (K) residues of fibrin. The formation of plasmin is enhanced by a positive feedback loop. New C-terminal lysine residues generated after limited plasmin cleavage provide new binding sites for continued tPA and plasminogen binding. TAFIa cleaves off the C-terminal lysine residues from partially degraded fibrin and thereby eliminates/decreases the fibrin cofactor function in the tPA/plasminogen/fibrin complex, inhibiting further formation of plasmin and the degradation of the fibrin clot.

TAFI = thrombin-activatable fibrinolysis inhibitor; tPA = tissue plasminogen activator; TAFIa = activated TAFI

α_2-M can operate as a second line of defense to inactivate remaining PLN. Antithrombin and α_1-antitrypsin have some antiplasmin activity in vitro but are probably minimally significant in vivo. C-1 inhibitor is thought to play a role in controlling contact phase-dependent fibrinolysis.[29]

CASE STUDY *(continued from page 644)*

9. Does any evidence exist to indicate a problem with Shawn's fibrinolytic system?

CONTROL OF HEMOSTASIS

An important aspect of coagulation involves the interactions and amplification loops required to get the burst of thrombin needed to form a stable clot. The dynamic fibrin formation process is normally limited to the site of vascular injury. However, the disruptive force of blood flow presents an extraordinary problem for regulating hemostasis. Activated factors and/or platelets must be kept at the site of injury and must be controlled so that blood remains fluid in the uninvolved vessels. Cellular localization and plasma protease inhibitors are essential in confining the coagulation reactions to sites of injury and turning off the process so that excessive clotting does not occur. Thus, physiologic mechanisms involved in controlling hemostasis include the control of blood flow, liver clearance of activated proteins, negative and positive feedback of activated clotting factors, and the protease inhibitors.

Blood Flow

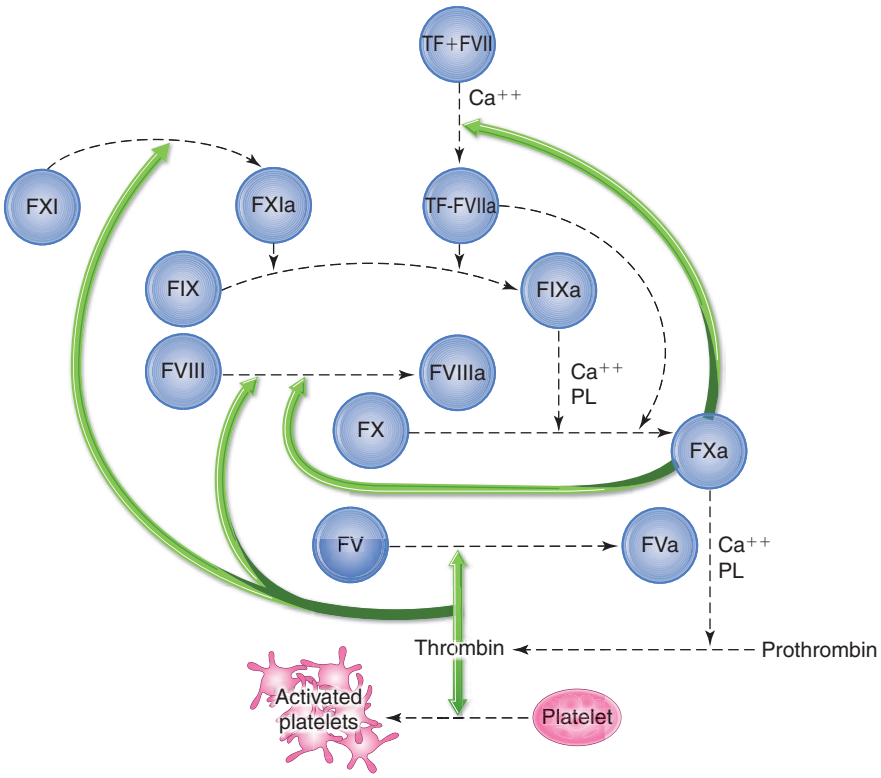
Vasoconstriction and activation of clotting factors are necessary for clot formation to begin. Vessel constriction initially enhances clot formation by slowing blood flow through the injured vessel. As blood pools, creating an area of stasis, platelets and coagulation factors are brought into proximity with the vessel wall, promoting the initiation of primary and secondary hemostasis. Neither stasis alone nor the activation of circulating coagulation factors in flowing blood results in clot formation. Vasoconstriction of the injured vessel is an important initial step for adequate fibrin formation. Return to normal blood flow through an area of injury then limits coagulation by diluting the concentration of activated factors. Activated factors carried away from the fibrin clot are bound by inhibitory proteins with loss of their coagulant potential.

Liver Clearance

The liver is the site of production of many clotting factors, making it a vital organ for normal hemostasis. The liver removes activated coagulation factors complexed with their inhibitors from the circulation as well as PLN-antiplasmin complexes (PAP) and FDPs. A major receptor for removal of these complexes is the LRP receptor found on hepatocytes and liver macrophages. Liver disease can result in hemorrhage from decreased production of coagulation factors. It can also contribute to systemic fibrinolysis or thrombosis associated with the failure to remove activated proteases.

Positive Feedback Amplification

The hemostatic system has several positive feedback mechanisms. Some of the most important are (1) thrombin, which, as a major activator of platelets, promotes the release of platelet FVa and the exposure of negatively charged phospholipid surfaces used for assembly of coagulation protein complexes, (2) thrombin activates FVa, FVIIIa, FXIa, and FXIIIa, (3) FXa feeds back to activate FVII, and (4) FXa has limited ability to activate FVIII in a reaction that can be important before significant amounts of thrombin are produced (Figure 32-17 ■).



■ **FIGURE 32-17** Positive feedbacks in coagulation. Heavy arrows indicate positive feedbacks. Shaded boxes indicate targets.

Negative Feedback Inhibition

Some activated factors have the potential to destroy other factors in the coagulation cascade. This process of feedback inhibition limits further enzyme production and dampens the coagulation process. Thrombin has the ability to activate FVa and FVIIIa and at higher concentrations can inactivate them via activated protein C (APC). FXa first activates FVIIa and then, through the action of tissue factor pathway inhibitor (TFPI), is itself inactivated in a reaction that requires FVIIa/TF. Fibrin, the end product of the cascade, also indirectly controls clotting. Fibrin has a strong affinity for thrombin, and once thrombin is adsorbed onto the fibrin meshwork it is very slowly released, limiting the amount of thrombin available to cleave more fibrinogen to fibrin. In addition, FDPs produced by PLN digestion function as inhibitors of fibrin formation by interfering with the conversion of fibrinogen to fibrin and the polymerization of fibrin monomers.

Biochemical Inhibitors

Naturally occurring inhibitors are soluble plasma proteins that regulate the enzymatic reactions of serine proteases. They prevent the initiation or amplification of the coagulation cascade (Table 32-5 ★; Figure 32-18 ■). The natural inhibitors include:

1. Antithrombin (AT)
2. Heparin cofactor II (HCII)
3. Protein C (PC) and protein S (PS)
4. TFPI
5. Protein Z-dependent protease inhibitor (ZPI) and protein Z (PZ)
6. α_2-M
7. α_1-Antitrypsin (α_1-protease inhibitor [α_1PI])
8. C1-esterase inhibitor (C1-INH)
9. TAFI (see the section "Inhibitors of Fibrinolysis")
10. α_2-AP (see the section "Inhibitors of Fibrinolysis")

★ **TABLE 32-5** Summary of Naturally Occurring Inhibitors

Component	Description	Role	Concentration in Plasma	T1/2	Chromosome (gene symbol)[a]
Protein C (PC)	Two-chain glycoprotein MW 62,000	Serine protease: FVa and FVIIIa inactivation	4–6 mcg/mL 60 nM	6 hr	2 (*PROC*)
Protein S (PS)	Single-chain glycoprotein MW 64,000	Cofactor for APC inactivation of FVa and FVIIIa	20–25 mcg/mL 300 nM	42 hr	3 (*PROS1*)
Thrombomodulin (TM)	Transmembrane protein MW 450,000	Cofactor/modulator: EC receptor for thrombin; cofactor for thrombin activation of PC; stimulator of EC to release tPA and EDRF	0.02 mcg/mL	—	20 (*THBD*)
Antithrombin (AT)	Single-chain glycoprotein MW 58,000	Serpin: inhibitor of thrombin, factors Xa, IXa XIa, XIIa, kallikrein, plasmin, tPA	150 mcg/mL 2500 nM	61–72 hr	1 (*SERPINC1*)
Heparin cofactor II (HCII)	Glycoprotein MW 66,000	Serpin: inhibitor of thrombin	40–70 mcg/mL 1000 nM	60 hr	22 (*SERPIND1*)
C1-esterase inhibitor (C-INH)	Single-chain glycoprotein MW 105,000	Serpin: inhibitor of kallikrein, plasmin, C1, FXIIa, FXIa	180 mcg/mL 1700 nM	70 hr	11 (*SERPING1*)
Protein C inhibitor (PAI-3)	Single-chain protein MW 57,000	Serpin: inhibitor of APC	1.0 mcg/mL 100 nM	23 days	14 (*SERPINA5*)
α_1-antitrypsin (α_1-proteinase inhibitor, α_1PI)	Protein MW 55,000	Serpin: inhibitor of FXIa, thrombin, kallikrein, plasmin, tPA	2500 mcg/mL 45,000 nM	144 hr	14 (*SERPINA1*)
Tissue factor pathway inhibitor (TFPI)	Single-chain glycoprotein MW 43,000	Inhibitor of FVIIa and FXa	0.07 mcg/mL 1.6 nM	1–2 min	2 (*TFPI*)
α_2-macroglobulin (α_2-M)	Dimeric protein MW 725,000	Proteinase inhibitor: inhibitor of kallikrein, plasmin, thrombin, tPA	2500 mcg/mL 3000 nM	—	12 (*A2M*)
Protein z-dependent protease inhibitor (ZPI)	Single-chain glycoprotein MW 72,000	Serpin: inhibitor of FXa with cofactor Protein Z; inhibitor of FXI	1.0–1.6 mcg/mL 20 nM	—	14 (*SERPINA10*)
Protein Z	Vitamin K-dependent glycoprotein MW 62,000	Cofactor for ZPI inhibition of FXa	2–3 mcg/mL 50 nM	60	13 (*PROZ*)

Serpin = serine protease inhibitor; tPA = tissue plasminogen activator; MW = molecular weight in Da.; APC = activated protein C; EC = endothelial cells; PAI = plasminogen activator inhibitor; EDRF = endothelium derived relaxing factor

[a]Gene symbol approved by the HUGO Gene Nomenclature Committee Protein C (PC)

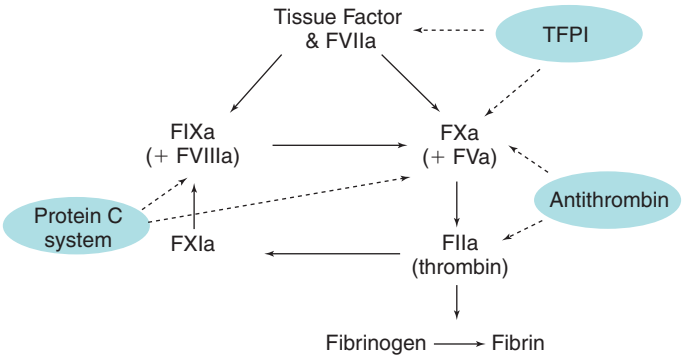


FIGURE 32-18 Major naturally occurring inhibitors of coagulation. Solid lines indicate proteolytic pathways. Dashed lines show direction of inhibition. Shaded ovals indicate inhibitors.

TFPI = tissue factor pathway inhibitor

Antithrombin, α_1-antitrypsin, heparin cofactor II, C1-inhibitor, ZPI, AP, and PAI-1 are members of the serpin family of **ser**ine **p**rotease **in**hibitors (serpins). Serpins inhibit their target enzymes in what has been described as a suicide-substrate inhibition mechanism in which they form a covalent complex with the enzyme's active site serine.[37] Conformational changes are induced in both the protease and the inhibitor, trapping the enzyme with the serpin and resulting in loss of activity of both proteins.

Antithrombin (AT)

Formerly called *AT III*, AT is a serpin protease inhibitor and is clinically the most important inhibitor of procoagulant serine proteases. The name *antithrombin* is misleading because AT can neutralize all serine proteases including thrombin; factors IXa, Xa, XIa, and XIIa; kallikrein; and plasmin. AT molecules circulating in the blood have limited inhibitory activity. AT forms a 1:1 complex with each target protease, but the reaction is slow in the absence of heparin or heparin-like molecules. Glycosaminoglycans (GAGs) such as heparin, heparan sulfate, or dermatan sulfate accelerate inhibition of the target proteases by three to four orders of magnitude. Originally, two antithrombin activities—progressive antithrombin (in the absence of heparin) and heparin cofactor (in the presence of heparin)—were described and were thought to result from two separate plasma proteins. Subsequently, both activities were shown to be associated with a single protein, AT.[38] Procoagulant proteases associated in surface-activating complexes (prothrombinase, tenase) are protected against AT/heparin inactivation.

AT is produced by hepatocytes and secreted into the plasma. In vivo, heparin is located in the granules of mast cells and basophils although under normal circumstances, it is not released from these cells into the circulation and cannot be detected in plasma. Although only small amounts of naturally occurring heparin are found in plasma, vascular endothelium is rich in heparin-like molecules, GAGs or heparan sulfate proteoglycans (HSPGs). These endothelial cell–associated proteins have heparan side chains with the correct carbohydrate sequences needed for AT recognition. Both thrombin and AT can bind to HSPGs, forming a ternary complex. Upon binding, AT undergoes a conformational change, making its reactive site more accessible to the active site serine of thrombin (or other protease targets) and increasing the rate of interaction several thousand-fold. The thrombin/AT (TAT) complex then dissociates from the proteoglycan, and the heparan sites are free to bind other thrombin and AT molecules. Dermatan sulfate, another GAG located in the vessel wall and the tissues, has little catalytic effect on AT but is a potent catalyst for heparin cofactor II. Vessel wall HSPGs bind and localize plasma AT, which contributes to the anticoagulant and antithrombotic properties of the endothelium by inhibiting free thrombin and other activated proteases in the plasma.

Heparin used as an anticoagulant consists of molecules that are structurally and functionally heterogeneous, ranging in size from 5000 to 30,000 Da. In commercial heparin preparations, only about one-third of the molecules have catalytic activity. Among naturally occurring heparin-like proteoglycans, <10% are active. Heparin inhibits platelet function by inhibiting VWF binding and reducing platelet adhesion to subendothelium and thus can produce hemorrhagic side effects if not monitored during therapeutic administration. Also, heparin can produce a mild thrombocytopenia (heparin-associated thrombocytopenia [HAT] Chapter 35), resulting from direct interactions between heparin and platelets. The low-molecular-weight (LMW) heparins (alternative therapeutic antithrombotic agents) can inactivate FXa but are less effective in inactivating thrombin. As the size of the heparin molecules decreases, the ratio of inactivation of FXa to thrombin increases. LMW heparins are also less likely to induce thrombocytopenia and HAT. Heparin has no action as an anticoagulant in the absence of AT. When AT levels are significantly reduced, patients can become unresponsive to antithrombotic therapy with heparin.

Heparin Cofactor II (HCII)

HCII is a second plasma protease inhibitor whose activity is accelerated by heparin. Unlike AT, HCII is not a broad-spectrum inhibitor. It inhibits thrombin but has little activity against other coagulation proteases (FXa, FIXa, FVIIa).[37] Because HCII's affinity for heparin is significantly less than that of AT, a higher concentration of heparin is needed to accelerate its thrombin inhibition. Therefore, HCII probably contributes a minimal anticoagulant effect in heparinized patients. However, it likely is involved in thrombin inhibition in extravascular locations because its activity (unlike that of AT) is accelerated significantly by extravascular dermatan sulfate. HCII may also play a role in protection from thrombosis during pregnancy, as plasma levels rise to ~150% during the third trimester.[39]

Protein C (PC) and Protein S (PS)

The PC pathway is a major inhibitory mechanism involved in controlling blood coagulation. Unlike the other inhibitory mechanisms directed at the proteases of the coagulation cascade, activated PC (APC) inhibits two of the nonproteolytic regulatory cofactors of coagulation: Va and VIIIa. APC cleavage of FVa at specific sites converts it to FVai, which totally lacks cofactor activity. Although FVIII is also a substrate of APC, its inactivation by APC may be of limited biologic significance because the intrinsic instability of FVIII is likely responsible for most of its loss of activity in vivo.[40]

PC and its cofactor, PS, are vitamin K-dependent proteins synthesized in the liver. PC circulates as a two-chain disulfide-linked zymogen containing 9 Gla residues. PS is a single-chain glycoprotein with 11 Gla residues. Unlike the other vitamin K-dependent factors, PS does not contain a serine protease domain and thus lacks protease activity. The protein C pathway is illustrated in Figure 32-19 ■.

When generated at the site of injury, thrombin does not remain tethered to the phospholipid surface that catalyzed its formation. As excess thrombin (T) accumulates, some binds to TM on adjacent endothelial cell (EC) surfaces. **Thrombomodulin (TM)** is an integral membrane protein named for its ability to alter the activity of thrombin from procoagulant to anticoagulant so that thrombin loses its ability to clot fibrinogen and activate FV, FVIII, and platelets, but instead rapidly activates PC in the presence of Ca^{++}.[41,42] TM binding seems to cause a conformational change in the thrombin molecule that accounts for its altered activity. APC is released from the thrombin/TM (T/TM) complex and in association with its cofactor PS, proteolytically inactivates FVa and FVIIIa in the presence of Ca^{++} and phospholipid. The precursor FV and FVIII molecules are resistant to the action of APC. The inactivation of these cofactors prevents sustained thrombin generation. Thus thrombin creates a self-dampening effect via activation of PC that limits the growth of the fibrin clot.

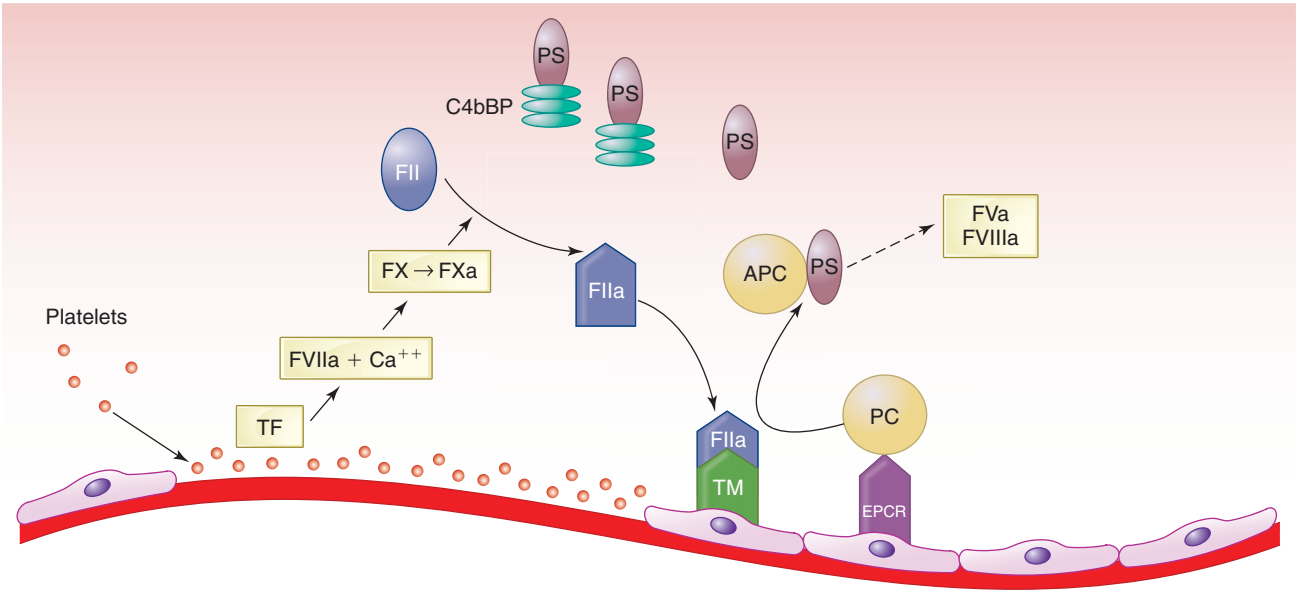
To function effectively, APC must interact with PS, forming a 1:1 complex in the presence of Ca^{++} and a phospholipid surface. PS circulates in the blood in two forms: (1) free PS (40% of the total circulating protein) and (2) protein that is noncovalently associated with the complement regulatory protein, C4b-binding protein (C4BP). The free form of PS is the only effective cofactor for APC. When bound to a cell surface, the complex of PC–PS is capable of inactivating FVa and FVIIIa. APC cleaves Arg 306 and Arg 506 in FVa, and the analogous Arg 336 and Arg 562 in FVIIIa. The inactive cleavage products of APC are referred to as *FVai* and *FVIIIai*. Inactivation of FVIIIa requires both PS and FVa as cofactors.[23] APC also produces a profibrinolytic effect, presumably because of the decreased generation of thrombin resulting in the lack of activation of TAFI.

An **endothelial cell protein C receptor (EPCR)** is found on the endothelial cells of larger vessels.[43] When EPCR binds PC, it augments PC activation by increasing the affinity of the T/TM complex for PC.[44] When activated by T/TM in the microcirculation, APC rapidly dissociates from the endothelial cells but dissociates more slowly from the endothelium of larger vessels because of binding to EPCR. When APC dissociates from EPCR, APC can bind PS and then inactivate FVa. APC bound to EPCR induces a profound anti-inflammatory effect, mediated at least in part via activation of protease-activated receptor-1 (PAR-1) on the EC membrane.[45] APC is neutralized by PC inhibitor (PCI, or PAI-3), α_1-antitrypsin, and α_2-M.

Deficiency of either PC or PS results in a tendency for thromboembolic disease. Patients have been described with normal amounts of PC and PS in their plasma, yet PC activation fails to inactivate FVa. This abnormality (FV Leiden), referred to as *APC resistance*, is the result of a mutation in one of the cleavage sites of FVa (Chapter 35).[46]

Tissue Factor Pathway Inhibitor (TFPI)

Originally called *extrinsic pathway inhibitor* or *lipoprotein-associated coagulation inhibitor*, **tissue factor pathway inhibitor (TFPI)** inhibits the FVIIa/TF complex, suppressing the activity of the extrinsic pathway. PS serves as a cofactor for TFPI's inhibition of FXa.[23]



■ **FIGURE 32-19** Protein C (PC) pathway. Thrombin (FIIa) forms on the vessel wall at the site of injury. Thrombomodulin (TM) on the endothelial cell forms a complex with thrombin. This complex activates PC, which then in association with free protein S (PS) inactivates FVa and FVIIIa.

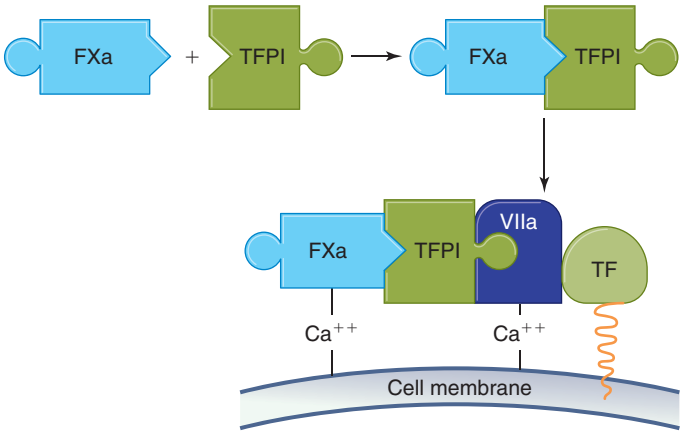
TF = tissue factor; FII = prothrombin; APC = activated protein C; C4BP = complement 4b binding protein; EPCR = endothelial cell protein C receptor

The microvascular endothelium is the major source of TFPI synthesis in vivo. A significant portion of TFPI is bound to heparan sulfate on the surface of ECs with most of the remainder in blood bound to low-density lipoproteins (LDL).[47] Heparin administration releases the EC-bound TFPI and raises the plasma level several-fold.[48] The range of plasma TFPI concentration varies highly among normal individuals. Plasma concentrations of TFPI activity vary with LDL levels because the interaction of TFPI with lipoproteins reduces the measurable anticoagulant activity.[49]

TFPI is unique among the protease inhibitors because it is a potent inhibitor of both FXa and FVIIa. First, TFPI binds to and inhibits the active site of FXa in the presence of Ca^{++} and procoagulant phospholipids. PS enhances this inhibition. Subsequently, this binary complex reacts with the FVIIa/TF complex, forming a quarternary compound (VIIa/TF/Xa/TFPI) on a membrane surface, inactivating both proteases[50] (Figure 32-20 ■).

The molecular basis for TFPI's capacity to neutralize two proteases simultaneously is the fact that TFPI has three potential inhibitor domains (Kunitz domains): The first domain binds to and inhibits the FVIIa/TF complex, and the second inhibits FXa. The third Kunitz domain lacks direct inhibitory activity but is involved in the PS-enhanced FXa inhibition.[50,51]

Because TFPI inhibition of FVIIa/TF requires FXa, the extrinsic pathway is not shut off until FXa is generated. Once FXa is produced, TFPI prevents continued activation of FX by the FVIIa/TF complex, and further FX activation must occur through the intrinsic pathway by the FIXa/FVIIIa complex. Thus, whereas initial FXa activation occurs via the extrinsic pathway, sustained activation requires FIXa activation via the intrinsic pathway.[52] This fact explains the severe bleeding associated with factor deficiencies of the intrinsic pathway. Deficiencies of factors VIII and IX (Hemophilia A and B) will not permit sufficient activation of FXa to maintain normal hemostasis even in the event of tissue damage and TF generation.



■ **FIGURE 32-20** The inhibition of FVIIa/TF by tissue factor pathway inhibitor (TFPI) occurs as the result of a multistep process. First, TFPI binds to FXa and neutralizes it, resulting in a conformational change of TFPI that promotes binding of the FXa/TFPI complex with the FVIIa/TF complex in a calcium-dependent reaction.

α_2-Macroglobulin (α_2-M)

The glycoprotein α_2-M is capable of inhibiting several serine proteases including thrombin, FXa, plasmin, and kallikrein. The inhibition rate is relatively slow when compared with other inhibitors, and protease activity is not completely neutralized. However α_2-M is thought to serve as a secondary or backup inhibitor for many coagulant and fibrinolytic enzymes.

Widely distributed in the body, the concentration of α_2-M changes with age with the highest levels in infants and children. It also can be elevated in pregnancy, in women using oral contraceptives, and in a number of disorders. Following initial binding to the target enzyme, α_2-M undergoes a conformational change, essentially trapping the enzyme within the inhibitor and preventing binding to its substrate. However, the catalytic site of the protease is left intact. This suggests that α_2-M can function primarily as a clearance mechanism for serine proteases rather than as an actual inhibitor of enzymatic activity. The α_2-M/protease complexes are rapidly cleared from plasma via binding to LRP receptors in the liver.

α_1-Antitrypsin (α_1-Protease Inhibitor [α_1-PI])

The glycoprotein α_1-PI has the capacity to inhibit a number of proteases and is the major inhibitor of FXIa.[53] Its activity is thought to be more important at the tissue level, particularly in its role as an inhibitor of neutrophil elastase.

C1-Esterase Inhibitor (C1-INH)

The C1-INH was first recognized as an inhibitor of the esterase activity of C1 from the complement cascade. It also inhibits the contact system proteases FXIIa, FIXa, kallikrein, and plasmin. C1-INH is the major plasma protease inhibitor of FXIIa and kallikrein, accounting for >90% of the plasma inhibitory activity.

Protein Z (PZ) and Protein Z-Dependent Protease Inhibitor (ZPI)

PZ is a vitamin K-dependent protein that contains 13 Gla residues. Like PS, PZ lacks a catalytic domain and serves as a cofactor, markedly enhancing the inhibitory function of ZPI.[23] ZPI is a plasma serpin that inhibits FXa in a PZ-dependent manner. It also inhibits FXIa in the absence of PZ but does not seem to inhibit other coagulation proteins.[15] The physiologic importance of ZPI and PZ in the in vivo regulation of coagulation is still unclear.

☑ CHECKPOINT 32-9

Why are naturally occurring inhibitors important in the hemostatic mechanism?

CASE STUDY (continued from page 653)

10. Why were liver function tests done on Shawn?
11. What is the significance of normal results in a patient with hemostatic disease?

PHYSIOLOGIC HEMOSTASIS

Although the coagulation proteins are found primarily in the plasma, coagulation is not a humoral or liquid-phase process but a cell-based system. Also, although it has been traditional to divide the coagulation mechanism into intrinsic and extrinsic pathways, it is now accepted that the two pathways do not operate independently of each other. TF is considered the key initiator of coagulation in vivo, and TF/VIIa can activate both FX and FIX, generating both "intrinsic" and "extrinsic" Xase activity (Figure 32-21 ■). This revised understanding of physiologic hemostasis has been referred to as the **tissue factor pathway**.

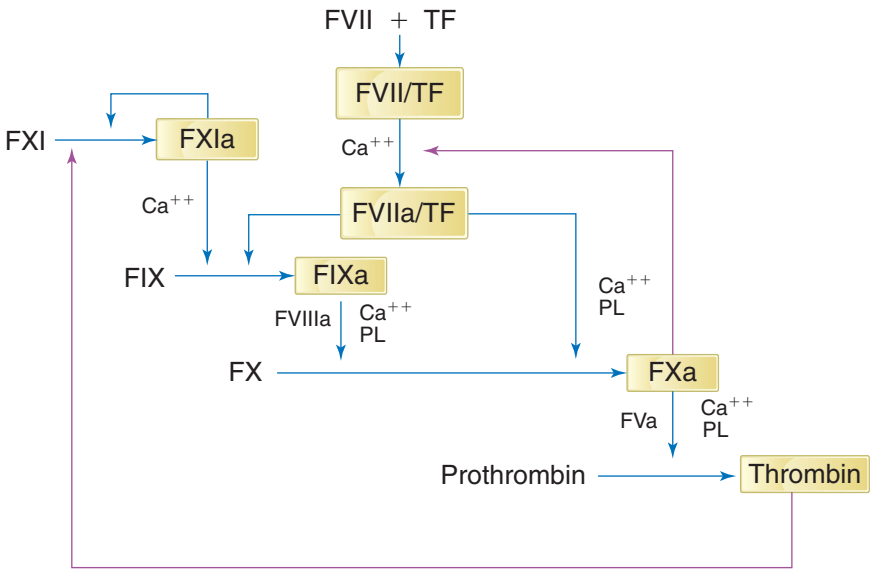
TF is not normally expressed on cells in contact with blood but is found on the surface of a variety of cell types outside the vasculature. Upon injury, cells expressing TF are exposed to the blood. Membrane-bound TF binds FVII or FVIIa with high affinity, anchoring the complex to the site of injury. FVIIa/TF then activates FX to FXa, and the common pathway continues. The extrinsic activation of FX would seem to make the activation of FX by the intrinsic Xase complex (FIXa, FVIIIa, PL, and Ca^{++}) unnecessary, but clinical observations have demonstrated the absolute necessity of these factors for normal hemostasis. Patients with FVIII and FIX deficiencies have major bleeding problems (Chapter 34) as do patients with severe deficiencies of factors II, V, VII, and X. The observation that FVIIa/TF also activates FIX to IXa demonstrated that extrinsic pathway activation could result in activation of FX through both "intrinsic" and "extrinsic" mechanisms (Figure 32-21). The roles of TFPI (see below) and the positive feedback effects of thrombin also contribute to explaining physiologic coagulation.

When coagulation is triggered, a series of events described as the *initiation*, *propagation*, and *termination* phases of coagulation occurs. Trace amounts of FVIIa are found in the circulation at all times and are not reactive with plasma protease inhibitors.[50] Coagulation is initiated when TF-bearing cells are exposed to blood at a site of vascular injury. The TF binds FVII or FVIIa, and the FVIIa/TF complex subsequently binds and activates FX and FIX on the surface of the TF-bearing cells. The result is the generation of a small amount of thrombin, which may or may not be sufficient to induce fibrin formation. However, it is sufficient to set in motion the events that will result in a subsequent burst of thrombin generation. Importantly, the TF-bound FVII is rapidly converted to FVIIa/TF by proteases (FVIIa/TF, FXa, thrombin) generated during the initiation phase.[3]

The small amounts of thrombin formed during the initiation phase launch a series of events associated with the multiple roles of thrombin in hemostasis that culminate in the acceleration or propagation of the coagulation mechanism. These events include the activation of (1) platelets, (2) FV, FVIII, and FXIII, and (3) FXI, IX, and X. The FXa produced on the TF-bearing cell is almost immediately inhibited by TFPI, which quickly inhibits the FVIIa/TF complex. Formation of thrombin through the "extrinsic" pathway is transient, and subsequent propagation of the coagulation cascade occurs on the surface of activated platelets (activated by either the initial injury or thrombin generated during the initiation phase). Additional activation of FX and generation of thrombin occur through the "intrinsic Xase" by the FIXa/FVIIIa complex generated by FVIIa/TF activation of FIXa and thrombin activation of FXIa. The result is massive prothrombin activation and with the surge in thrombin generation, activation of PC and TAFI (via thrombin binding to TM).

TF is distributed throughout the body and is abundant in a variety of cell types. Thus, it is readily available to trigger the clotting cascade anywhere following vascular injury. This distribution pattern has been described as providing a protective "hemostatic envelope" surrounding the vasculature and organ structures.[50]

Once the platelet–fibrin clot has formed, the coagulation process must be terminated to prevent clot extension into noninjured areas of the vasculature. The coagulation reactions subside in the termination phase. Activated by the burst of thrombin generation in the propagation phase, the PC/PS/TM system inactivates FVa and FVIIIa, preventing the generation of additional thrombin. AT and TFPI bound to heparan sulfate molecules on intact endothelium adjacent to the area of injury inactivate proteases that venture beyond the injury area.



■ **FIGURE 32-21** Tissue factor (TF) initiation of the physiologic pathway by activation of FIX and FX. Purple lines indicate positive feedback activation of TF/FVII by Xa and activation of FXI by thrombin.

Circulating protease inhibitors directly inhibit proteases that escape into the fluid phase.

Patients with hereditary deficiencies of FXII, PK, and HK have a markedly prolonged partial thromboplastin time (PTT), suggesting an abnormality in the intrinsic pathway but do not have abnormal bleeding in vivo because in vivo hemostasis requires only FVIIa/TF activation of FIX and FX and thrombin activation of FXI. The severe bleeding seen in patients with deficiencies of FVIII or FIX occurs because of their inability to generate the "burst" of thrombin generation associated with the propagation phase of coagulation. In addition to failing to generate enough thrombin to produce an adequate fibrin clot, the thrombin generated is insufficient to activate TAFI and suppress fibrinolysis.

CASE STUDY *(continued from page 657)*

A specific assay for FVII activity was done on Shawn and both of his parents. His FVII activity was found to be 3% of that in normal plasma. Prothrombin and FX activities were normal. The FVII activity of Shawn's father and mother was 50% and 47%, respectively. (The reference interval of FVII activity is 70–120%.)

12. Do these findings explain the patient's bleeding history?

Summary

The hemostatic system functions to keep blood fluid within the vasculature and to prevent excessive blood loss upon vascular injury. Platelets, vascular endothelial cells, and numerous coagulation proteins interact to maintain a balance between bleeding and clotting in vivo. Primary hemostasis occurs through activation of platelets and results in the formation of an unstable platelet plug. The formation of fibrin (coagulation) subsequently reinforces this plug and stabilizes it. The fibrin formation occurs through a process called *secondary hemostasis*.

The process of fibrin formation is carefully controlled and limited to areas of damage within the vascular network. Localization of the response to the site of injury prevents widespread coagulation activation. Activation of the coagulation system occurs on phospholipid membrane surfaces (activated platelets) or on exposed subendothelial tissue at sites of vessel injury. Coagulation involves a series of sequential activations of inactive zymogens to active enzyme products. Classically, activation of the coagulation system has been described as being initiated via one of two possible pathways, the intrinsic and the extrinsic. Currently, however, it is believed that this distinction, although useful in discussing the system, is probably not physiologically relevant. In vivo, FVIIa/TF activation of FX and FIX is believed to initiate coagulation.

Each sequential activation step in the coagulation cascade involving the generation of an active clotting enzyme is modified and/or controlled by cofactors that accelerate the activation and inhibitory mechanisms that slow activation. The net result of these two opposing forces is a well-balanced physiologic process designed to control activation of the system to an appropriate degree. The final activation step results in the formation of the enzyme thrombin, which is responsible for converting the soluble plasma protein, fibrinogen, into an insoluble state, fibrin.

Once the fibrin clot has been formed and hemostasis achieved, repair of the damaged vascular tissue is initiated. The final step in the process requires the dissolution of the fibrin clot by the plasma fibrinolytic system once it is no longer needed. Like clotting, fibrinolysis involves the activation of an inactive proenzyme precursor (plasminogen) to the active enzyme plasmin. Both activators (PAs) and inhibitors (PAIs and α_2-AP) also help regulate this process.

Review Questions

Level I

1. Which of the following is/are involved in hemostasis? (Objective 1)

   A. only blood clotting

   B. clot formation, clot dissolution, vessel repair

   C. only blood clotting and bleeding

   D. only keeping blood in the fluid state

2. Which of the following describes the events involved in secondary hemostasis? (Objective 2)

   A. lead to the formation of a chemically stable fibrin clot

   B. usually occur independently of primary hemostasis

   C. are uncontrolled

   D. occur in a random fashion

3. Which factor is known as the *antihemophilic factor?* (Objective 3)
   A. FIX
   B. FXI
   C. FVIII
   D. FXII
4. The prothrombin group includes which of the following coagulation proteins? (Objective 4)
   A. factors I, V, VIII, XIII
   B. PK, HK, FXI, FXII
   C. factors II, VII, IX, X
   D. factors I, II, V, X
5. Which of the following function as cofactors in hemostasis? (Objective 6)
   A. fibrinogen, prothrombin
   B. FVa, FVIIIa
   C. factors IXa, XIa, XIIa
   D. platelet phospholipid, Ca^{++}
6. What activates the extrinsic pathway? (Objective 7)
   A. exposure to negatively charged surfaces
   B. contact with tissue factor
   C. the intrinsic pathway
   D. FXIIa
7. Which factors are involved in the initial activation of the coagulation system and require contact with a negatively charged surface for their activation? (Objectives 7, 8)
   A. factors II, V, VII, X
   B. factors XII, XI, PK, HK
   C. factors II, VII, IX, X
   D. factors I, V, VIII, XIII
8. Which of the following is *not* involved in the fibrinolytic system? (Objective 10)
   A. plasminogen activator inhibitors
   B. plasmin
   C. FXa
   D. FXII, kallikrein
9. Which of the following is/are end products in the breakdown of fibrin? (Objective 11)
   A. fragment X
   B. fragments Y and D
   C. fragments D and E
   D. fibrin monomer
10. Which of the following pairs the correct protease with its inhibitor? (Objective 12)
    A. thrombin–antithrombin
    B. tissue factor–TFPI
    C. heparin–heparin cofactor II
    D. fibrin–TAFI

Level II

1. Hemostasis depends on the balance of which of the following? (Objective 1)
   A. the interaction of blood vessels, platelets, and coagulation proteins
   B. the interaction between platelets and coagulation proteins
   C. the interaction between blood vessels and coagulation proteins
   D. the rate of complex formation
2. Which statement most accurately describes the domains of coagulation proteins? (Objective 2)
   A. The catalytic region distinguishes each of them from trypsin.
   B. The catalytic region gives each protein its own identity.
   C. The noncatalytic regions are identical in all coagulation proteins.
   D. The domains impart specificity and recognition to control hemostasis.
3. Which of the following is true concerning the formation of protein complexes that occur during coagulation? (Objective 3)
   A. slows the rate of reaction
   B. does not require the presence of Ca^{++}
   C. is not necessary for normal coagulation
   D. localizes the reaction to the site of injury
4. Which of the following describes the contact factors? (Objective 4)
   A. have a significant role in initiating hemostasis in vivo
   B. cause a serious bleeding problem when defective or deficient
   C. contribute to fibrinolysis, inflammation, and complement activation
   D. can be assayed by the prothrombin time

5. The role of ADAMTS-13 in hemostasis is to: (Objective 6)
   A. activate protein C to activated protein C
   B. cleave ULVWF to multimers normally found in the circulation
   C. serve as a receptor for uPA on hepatocytes
   D. inhibit activated hemostatic proteases
6. What is the hepatic cell receptor that binds and clears tPA/PAI-1, plasmin/AP, and uPA/PAI-1 complexes from the circulation? (Objective 6)
   A. UPAR
   B. LRP
   C. annexin-2
   D. TM
7. Which of the following is a procoagulant function of thrombin? (Objective 5)
   A. activate FXII
   B. inactivate protein C
   C. suppress platelet activation
   D. activate FV and FVIII
8. What is needed for fibrinolysis to occur at a physiologically significant rate? (Objective 7)
   A. tPA must bind to fibrin.
   B. Circulating plasminogen must be activated to plasmin.
   C. PAI-1 must be inhibited by α_2-antiplasmin.
   D. Plasminogen must be bound to fibrinogen.
9. Which is true of TFPI? (Objective 8)
   A. is found in platelets
   B. is unique because two different proteases are inhibited at the same time
   C. does not require Ca^{++}
   D. inhibits the entire coagulation cascade by inhibiting the extrinsic pathway
10. The following results were reported on a 10-year-old male with a history of bleeding problems: prothrombin time: 29.5 seconds (control, 12.5 seconds), partial thromboplastin time: 51.0 seconds (control, 55.4 seconds). What do these results indicate? (Objective 9)
    A. An FVII defect or deficiency exists.
    B. A defect in the intrinsic system exists.
    C. The child has hemophilia A.
    D. Platelet testing should be done.

Companion Resources

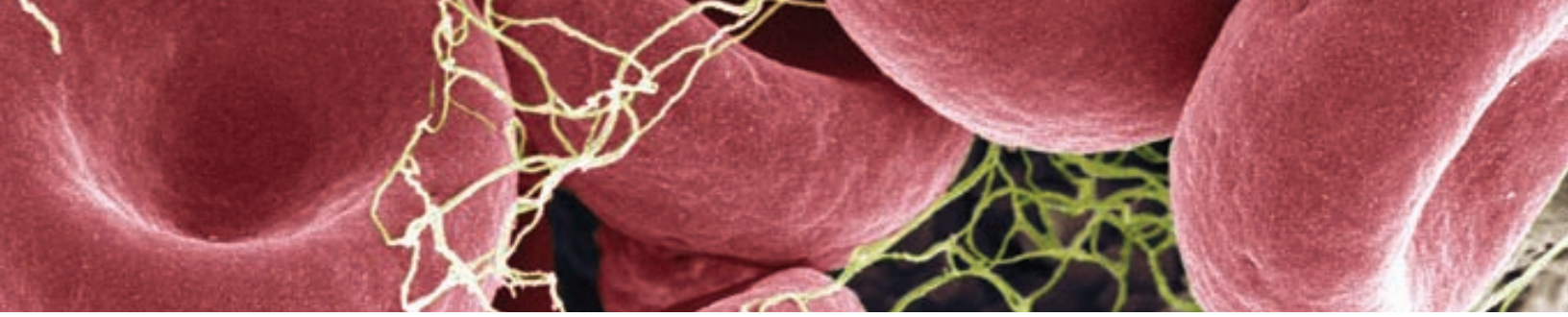
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. MacFarlane RG. An enzyme cascade in the blood clotting mechanism and its function as a biochemical amplifier. *Nature*. 1991;202:498–99.
2. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. *Science*. 1964;145:1310–12.
3. White GC, Marder VJ, Schulman S et al. Overview of basic coagulation and fibrinolysis. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:103–9.
4. Wright IS. The nomenclature of blood clotting factors. *Thromb Diath Haemorrh*. 1962;27:381–8.
5. Rapaport SI. Blood coagulation and its alterations in hemorrhagic and thrombotic disorders. *West J Med*. 1993;158:153–61.
6. Furie B, Furie BC. The molecular basis of blood coagulation. *Cell*. 1998;53:505–18.
7. Scully MF. The biochemistry of blood clotting: the digestion of a liquid to form a solid. *Essays Biochem*. 1992;27:17–36.
8. Nesheim ME, Taswell JB, Mann KG. The contribution of bovine factor V and factor Va to the activity of prothrombinase. *J Biol Chem*. 1979;245:10,952–62.
9. Renné T. The factor XII-driven plasma contact system. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:242–53.
10. Ratnoff OD. The biology and pathology of the initiation of coagulation. In: Broom EB, Moore CV, eds. *Progress in Hematology*. New York: Grune & Stratton; 1966:204–45.
11. Colman RW. Contact activation (kallikrein-kinin) pathway: multiple physiologic and pathophysiologic activities. In: Colman RW, Marder VJ, Clowes AW et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2006:107–30.
12. Walsh PN, Gailani D. Factor XI. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:233–41.
13. Kaufman RJ, Fay PJ, Popolo L et al. Factor V and factor VIII. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:179–96.

14. Brinkhous KM, Sandberg H, Garris JB et al. Purified human factor VIII procoagulant protein: comparative hemostatic response after infusions into hemophilic and von Willebrand disease dogs. *Proc Natl Acad Sci U S A*. 1985;92:9752–56.
15. Chung DW, Xu W, Davie EW. The blood coagulation factors and inhibitors: their primary structure, complementary DNAs, genes and expression. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:110–45.
16. Haberichter SL, Montgomery RR. Structure and function of von Willebrand factor. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:197–207.
17. Wise RJ, Barr PJ, Wong PA et al. Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc Natl Acad Sci U S A*. 1990;87:9378–82.
18. Fijikawa K, Suzuki H, McMullen B et al. Purification of human von Willebrand factor-cleaving protease. *Blood*. 2001;98:1662–66.
19. Dong JF, Moake JL, Nolasco L et al. ADAMTS-13 rapidly cleaves newly secreted ultra-large von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002;100:4033–39.
20. Morrissey JH, Macik BG, Neuenschwander PF et al. Quantitation of activated factor VII levels in plasma using tissue factor mutant selectively deficient in promoting factor VII activation. *Blood*. 1993;81:734–44.
21. Radcliffe R, Nemerson Y. Activation and control of factor VII by activated factor X and thrombin: isolation and characterization of a single chain form of factor VII. *J Biol Chem*. 1975;250:388–95.
22. Tracy PB, Eide LL, Bowie EJ et al. Radioimmunoassay of factor V in human plasma and platelets. *Blood*. 1982;60:59–63.
23. Vadivel K, Schmidt AE, Marder VJ et al. Structure and function of vitamin K-dependent coagulant and anticoagulant proteins. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:208–32.
24. Brox JH, Osterus B, Bjorklid E et al. Production and availability of thromboplastin activity in endothelial cells: the effects of thrombin, endotoxin, and platelets. *Br J Haematol*. 1984;57:239–46.
25. Doolittle RF. The structure and evolution of vertebrate fibrinogen. *Ann N Y Acad Sci*. 1983;408:13–27.
26. Fowler WE, Erickson HP. Trinodular structure of fibrinogen: confirmation by both shadowing and negative stain electron microscopy. *J Mol Biol*. 1979;134:241–49.
27. Yee VC, Pratt KP, Cote HC et al. Crystal structure of a 30kDa C-terminal fragment from the gamma chain of human fibrinogen. *Structure*. 1997;5:125–38.
28. Duval C, Philippou H, Ariens RAS. Factor XIII. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:272–85.
29. Mutch NJ, Booth NA. Plasminogen activation and regulation of fibrinolysis. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:314–33.
30. Hajjar KA, Francis CW. Fibrinolysis and thrombolysis. In: Lichtman MA, Beutler A, Kipps TJ et al., eds. *Williams Hematology*, 7th ed. New York: McGraw-Hill; 2006:2089–115.
31. Rijken DC, Hoylaerts M, Collen D. Fibrinolytic properties of one-chain and two-chain human extrinsic (tissue-type) plasminogen activator. *J Biol Chem*. 1982;257:2920–925.
32. Sepanova V, Bobik A, Bibilashvily R et al. Urokinase plasminogen activator induces smooth muscle cell migration: key role of growth factor-like domain. *FEBS Lett*. 1997;414:471–4.
33. Lijnen HR, Zamarron C, Blaber M et al. Activation of plasminogen by prourokinase. I. Mechanism. *J Biol Chem*. 1986;261:1253–58.
34. Scanu AM, Lawn RM, Berg M. Lipoprotein (a) and atherosclerosis. *Ann Intern Med*. 1991;115:209–18.
35. Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *J Biol Chem*. 1996;271:16603–608.
36. Wang W, Boffa MB, Bajzar L et al. A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activatable fibrinolysis inhibitor. *J Biol Chem*. 1998;273:176–81.
37. Bock SC. Antithrombin and the serpin family. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:286–99.
38. Abildgaard U. Highly purified antithrombin III with heparin cofactor activity prepared by disc electrophoresis. *Scand J Clin Lab Invest*. 1968;125:89–91.
39. Andrew M, Mitchell L, Berry L et al. An anticoagulant dermatan sulfate proteoglycan circulates in the pregnant woman and her fetus. *J Clin Invest*. 1992;89:321–26.
40. Mosnier LO, Griffin JH. Protein C, Protein S, thrombomodulin, and the endothelial protein C receptor pathways. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:300–13.
41. Esmon CT, Esmon NL, Harris K. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *J Biol Chem*. 1982;257:7944–47.
42. Esmon NL, Carroll RC, Esmon CT. Thrombomodulin blocks the ability of thrombin to activate platelets. *J Biol Chem*. 1983;258:12238–242.
43. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem*. 1994;269:26486–91.
44. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS et al. The endothelial cell protein C receptor augments protein C activation by the thrombin–thrombomodulin complex. *Proc Natl Acad Sci U S A*. 1996;93:10212–216.
45. Hajjar KA, Esmon NL, Marcus AJ et al. Vascular function in hemostasis. In: Lichtman MA, Beutler A, Kipps TJ et al., eds. *Williams Hematology*, 7th ed. New York: McGraw-Hill; 2006:1715–37.
46. Dahlbeck B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. *Proc Natl Acad Sci U S A*. 1994:91:1396–400.
47. Hansen JB, Huseby NE, Sandset PM et al. Tissue factor pathway inhibitor and lipoproteins: evidence for association with and regulation by LDL in human plasma. *Arterioscler Thromb Vasc Biol*. 1995;15:879–85.
48. Sandset P, Abildgaard U, Larsen M. Heparin induces release of extrinsic coagulation pathway inhibitor (EPI). *Thromb Res*. 1988;50:803–13.
49. Caplice NM, Panetta C, Peterson TE et al. Lipoprotein (a) binds and inactivates tissue factor pathway inhibitor: a novel link between lipoproteins and thrombosis. *Blood*. 2001;98:2980–987.
50. Morrissey JH, Broze GJ. Tissue factor and the initiation and regulation (TFPI) of coagulation. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:163–78.
51. Broze GJ, Girard TJ, Novotny WF. The lipoprotein-associated coagulation inhibitor. *Prog Hemost Thromb*. 1991;10:243–68.
52. Hoffman M, Monroe DM, Oliver JA et al. Factors IXa and Xa play distinct roles in tissue factor-dependent initiation of coagulation. *Blood*. 1995;86:1794–1801.
53. Scott CF, Schapira M, James HL et al. Inactivation of factor XIa by plasma protease inhibitors: predominant role of alpha 1-protease inhibitor and protective effect of high molecular weight kininogen. *J Clin Invest*. 1982;69:844–52.



33 Disorders of Primary Hemostasis

BARBARA A. O'MALLEY, MD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define and differentiate among *thrombocytopenia*, *thrombocytosis*, and *thrombocythemia* and state an expected range of platelet count in each.
2. Define and differentiate among *petechiae*, *purpura*, *ecchymoses*, *hematoma*, and *easy bruisability*.
3. Identify laboratory tests that can be ordered to screen for abnormalities of the hemostatic system.
4. Explain the expected clinical consequences when a patient has an abnormality of platelets or blood vessels.
5. Correlate quantitative variations in the platelet count with disease manifestations.
6. Recognize hematologic disorders characterized by the presence of thrombocytopenia or thrombocytosis.
7. Describe the etiology, pathophysiology, and laboratory findings of the thrombocytopenias.
8. Differentiate primary (malignant) from secondary (reactive) thrombocytosis.
9. Explain the effect of aspirin and its duration on platelet function.
10. Identify the cause and describe the clinical and laboratory features of hereditary disorders of platelet function.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Categorize each specific disorder of hemostasis by body system affected (e.g., vasculature, platelets).
2. Predict the type of bleeding symptoms in patients with disorders of primary hemostasis.
3. Describe the expected symptomatology, etiology, pathophysiology, and laboratory test results in patients with disorders of the vasculature.
4. Organize thrombocytopenic and thrombocytosis conditions by etiology and pathophysiology and explain laboratory findings in each condition.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

5. Differentiate acute from chronic immune thrombocytopenia by significant clinical and laboratory data.
6. Explain the pathophysiology of thrombocytopenia and thrombocytosis in hematologic disorders.
7. Organize the hereditary and acquired qualitative platelet defects by etiology and pathophysiology, and predict the clinical and laboratory features.
8. Explain the biochemical mechanism of the effect of aspirin on platelet function and recommend a time frame for patients to refrain from taking aspirin and related anti-inflammatory drugs prior to platelet function testing.
9. Summarize the effect of aspirin, alcohol, and antibiotics on platelet function.

Key Terms

Bernard-Soulier syndrome (BSS)
Congenital amegakaryocytic thrombocytopenia (CAMT)
Congenital thrombocytopenia with radioulnar synostosis (CTRUS)
δ-storage pool disease
Disseminated intravascular coagulation (DIC)
Ecchymoses
Epistaxis
Fanconi anemia (FA)
Glanzmann thrombasthenia (GT)
Gray platelet syndrome (GPS) (α-storage pool disease)
Hematoma
Hemolytic uremic syndrome (HUS)
Heparin-associated thrombocytopenia (HAT)
Heparin-induced thrombocytopenia (HIT)
Immune thrombocytopenia (ITP)
Ischemia
May-Heggelin anomaly (MHA)
Necrosis
Neonatal alloimmune thrombocytopenia (NAIT)
Nonthrombocytopenic purpura
Petechiae
Primary thrombocytosis
Purpura
Recombinant factor VIIa (rFVIIa)
Quebec platelet disorder
Scott syndrome
Secondary (reactive) thrombocytosis
Thrombocytopenia with absent radii (TAR)
Thrombotic thrombocytopenic purpura (TTP)
Vasculitis
Wiskott-Aldrich syndrome (WAS)
X-linked thrombocytopenia (XLT)
X-linked thrombocytopenia with thalassemia (XLTT)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Hemostasis: Describe how a blood clot forms after an injury, especially the role of platelets in cessation of bleeding. (Chapter 31)
- Immunology: Define and describe antigen/antibody reactions, classes of immunoglobulins, and the process of immune complex formation. (Chapter 8)
- Immune hemolytic anemia: Summarize the pathophysiology of the immune hemolytic anemias. (Chapter 19)
- Correlate the automated platelet count with the platelet count estimate on a peripheral blood smear. (Chapters 9, 10)
- Identify artifacts that can cause spuriously increased or decreased automated platelet counts. (Chapters 9, 10)

Level II

- Hemostasis: Correlate the functions of the blood vessels, platelets, and coagulation factors in forming a blood clot. (Chapters 31, 32)
- Neoplastic leukocyte disorders: Summarize the consequences of malignant diseases of the bone marrow, particularly as they relate to platelet production. (Chapters 23–28)
- Cytokines: Describe how cytokines and growth factors regulate blood cell production. (Chapter 4)
- Flow cytometry: Recognize and correctly utilize the CD nomenclature of cellular antigens. (Chapter 40)

CASE STUDY

We will address this case study throughout the chapter.

Mohammed, a 15-year-old male from Saudi Arabia, was admitted to the emergency room after an automobile accident with several superficial cuts and bruises to his head and arms. He was bleeding profusely and more severely than would be expected from the nature of his wounds.

Consider possible causes of this abnormal bleeding and the laboratory tests that might be used to differentiate and diagnose the cause.

OVERVIEW

This chapter is the first of two that describe abnormalities of the hemostatic system that result primarily in bleeding. It begins with the general clinical and laboratory aspects of hemostatic disorders, followed by a discussion of defects in primary hemostasis, including the vascular system and platelets. The pathophysiologic basis and clinical manifestations for each defect are presented, but the major emphasis is on laboratory involvement in the diagnosis and/or treatment of the conditions. It is important to correctly identify the cause of the hemostatic defect so that appropriate treatment or preventive measures can be implemented.

INTRODUCTION

As discussed in Chapters 31 and 32, hemostasis minimizes blood loss from disruptive injuries to blood vessels and prevents blood loss from intact vessels. The hemostatic response includes vasoconstriction of blood vessels, primary hemostatic plug formation by platelet activation, fibrin formation by activation of soluble plasma proteins, and the function of inhibitors that prevent inappropriate or excessive activation of hemostasis and regulate the system to allow activation only when and where it is needed. Adequate hemostasis depends on a large number of intricately balanced mechanisms. Abnormalities of one or more components in the process of clot formation (i.e., the blood vessels, platelets, or clotting factors) can lead to excessive bleeding. Failure to regulate excessive clot formation leads to thrombosis.

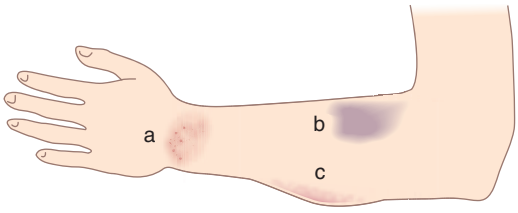
DIAGNOSIS OF BLEEDING DISORDERS

Clinical Manifestations of Bleeding Disorders

A patient with a clinically significant bleeding disorder presents to the physician with hemorrhagic symptoms. Bleeding symptoms can range from easy bruisability to life-threatening hemorrhage. The severity of the bleed is generally proportional to the severity and type of hemostatic defect.

The type of bleeding can indicate which component of the hemostatic system is defective. A defect in a component of primary hemostasis (vasculature, platelets) usually results in bleeding from the skin or mucous membranes, such as epistaxis (nose bleeds), gingival mucosa (gums), or menorrhagia (abnormal menstrual bleeding). Bleeding symptoms in patients with coagulation factor abnormalities, on the other hand, are usually internal, involving deeper tissues and joints.

Bleeding from subcutaneous blood vessels (capillaries) into intact skin can be visualized as petechiae, purpura, ecchymoses, or hematomas (Figure 33-1 ■). **Petechiae** are small red to purple spots in the skin <3 mm in diameter resulting from blood leakage through the endothelial lining of capillaries. Petechiae usually occur on the extremities because of the high venous pressure. When occurring spontaneously without trauma, they are painless. Several petechiae in one area can merge into a larger bruised area. Petechial lesions are characteristic of abnormalities of platelets and blood vessels and usually are not seen in coagulation factor disorders.



■ **FIGURE 33-1** Schematic drawing of bleeding manifestations in intact skin. **(a)** Petechiae. **(b)** Ecchymosis. **(c)** Hematoma.

Ecchymoses are bruises that are larger than 1 cm in diameter and are caused by blood escaping through the endothelium into subcutaneous tissue, commonly from a vessel larger than a capillary. They are red or purple when first formed and become yellowish green as they heal and hemoglobin degrades into bilirubin and biliverdin. Ecchymoses can appear spontaneously or with trauma and can be painful and tender. They can occur when abnormalities of blood vessels, platelets, or coagulation factors exist.

Intermediate lesions (>3 mm but <1 cm) are called **purpura**. A **hematoma** is formed when blood leaks from an opening in a vessel and collects beneath intact skin, forming a blue or purple slightly raised area. Hematomas can occur in any organ or tissue.

When ecchymoses and purpura are found in higher than normal numbers and with less than usual trauma, the condition is termed *easy bruisability*. Bleeding disorders can also be characterized by *excess bleeding*, which occurs from superficial cuts and scratches when platelets fail to form an effective primary hemostatic plug. Excess bleeding indicates that the bleeding is more prolonged and/or more profuse than normal for the patient or as compared with a normal person. Blood can escape from visceral organs into any body cavity or from mucous membranes into any body orifice. Frank bleeding is characteristic of both platelet and coagulation abnormalities.

Evaluation of a Patient with Abnormal Bleeding

Excessive bleeding can be caused by a local disruption of the vasculature, such as a bleeding ulcer, or by generalized failure of the hemostatic mechanism. In some cases, both are present, compounding the effect of the vascular disruption. Determining the cause of the bleeding and instituting proper treatment are important. Doing so requires obtaining an accurate medical history, performing a thorough physical examination, and ordering and interpreting the results of appropriate laboratory tests.

The patient's history should include:

- Age of onset of hemorrhagic symptoms
- Type of symptoms
- Family history
- Presence of other diseases
- Complete drug history (including over-the-counter drugs and herbal supplements)
- Exposure to toxins

The answers should enable the evaluation of whether a bleeding disorder exists, determine the probability of an inherited versus an acquired condition, and help define the affected portion of the hemostatic system. The age at which symptoms first appeared provides a clue to the disorder's etiology. Bleeding occurring at birth or shortly thereafter often indicates an inherited disorder, although onset later in life does not rule this out. Bleeding from the umbilical cord stump and/or from the circumcision site suggests a coagulation factor defect.

The persistence and severity of symptoms—whether occurring throughout life, a single event, or intermittently—is also informative.

Bleeding in excess of that expected from a tonsillectomy, tooth extraction, trauma, injury, surgery, or childbirth can provide clues.

Family history is helpful in determining whether other family members have had similar symptoms. A pedigree analysis can help to establish the pattern of inheritance (X-linked, or autosomal dominant or recessive). Patients with genetic abnormalities do not always have a positive family history because spontaneous (de novo) mutations occur in several hemostatic disorders.

The presence of an associated disease or malignancy, such as aplastic anemia, liver disease, uremia, infection, or leukemia must be considered. These conditions can be associated with secondary or acquired platelet disorders and/or coagulation defects.

A history of drug exposure is important to consider because many drugs are known to affect the hemostatic mechanism, and various drugs affect different portions of the system. Aspirin, chemotherapeutic drugs, Coumadin, and other anticoagulants are examples, but there are many others. The reader is referred to the Companion Resources for Chapter 36 and to other texts that provide extensive lists of specific drugs that have been implicated in acquired hemostatic defects.[1–3] Finally, past or present exposure to toxic chemicals such as benzene, insect sprays, and hair dyes should be investigated.

On physical examination, the type and sites of bleeding should be noted as well as whether the bleeding is from single or multiple sites and whether it is spontaneous or the result of trauma. Using the information from the history and physical examination, appropriate laboratory tests to confirm and classify the presence of abnormal hemostasis can be ordered.

Laboratory Evaluation of Abnormal Bleeding

Although laboratory testing plays an important role in evaluating a patient with a suspected hemostatic abnormality, it should never be a substitute for good clinical assessment of the patient. No single laboratory test can fully evaluate defective hemostasis, so initially a battery or group of screening tests is usually ordered. Based on their results, it can be determined whether a detectable abnormality of hemostasis does exist and the confirmatory tests necessary to define the disorder can be ordered. Initial screening tests include:

- Platelet count
- Prothrombin time (PT)
- Activated partial thromboplastin time (APTT)

The result of one or more of these tests is abnormal in many patients with hemostatic disorders, and they are often within the reference interval if the patient does not have a defect in the hemostatic system. However, some patients with disorders of hemostasis can have normal results in these initial three screening tests, necessitating additional testing. Also, screening tests are not helpful in predicting *bleeding risk* for the patient. In patients with disorders of primary hemostasis, the PT and APTT are usually normal, and the platelet count may or may not be abnormal.

When the results of these screening tests are normal in a patient with a history of clinically significant bleeding, a vascular disorder or functional platelet defect is likely. Confirmatory tests of platelet function can then be ordered. These include the closure time (measured by platelet function analyzer, PFA-100®), platelet aggregation tests, and flow cytometry for platelet antigens. The template bleeding time was historically used to evaluate platelet function and measured the time for an incision to stop bleeding (Chapter 36). It is rarely used today because it lacks reproducibility. The PFA-100 is an alternative to the bleeding time test. It provides automated assessment of platelet dysfunction by aspirating a blood sample through a capillary tube at high shear flow to simulate the hemodynamic conditions of platelet adhesion and aggregation at a vascular lesion. Using cartridges with membranes coated with collagen and adenosine diphosphate (ADP) or collagen and epinephrine, the blood encounters the coated membranes, triggering platelet adhesion, activation, and aggregation, leading to the occlusion of an aperture and cessation of blood flow. In the absence of abnormal results of platelet function testing, the possibility of a vascular disorder can be investigated.

Abnormality of the PT and/or APTT denotes a coagulation factor disorder (Chapter 34). Numerous confirmatory tests are available to specify the diagnosis (Chapter 36).

In some patients with mild hemostatic disease, the screening tests might not be sensitive enough to detect an abnormality. Other patients could have conditions that do not affect the screening tests. In such cases, further investigation using the information obtained from the clinical history and physical examination may be warranted.

Laboratory testing for the hemostatic system consists of a number of tests, each of which evaluates a specific part of the hemostatic system. It is important for a clinical laboratory professional to correlate and understand the significance of the results of all hemostasis tests and to correlate the results with the patient's history, if available. In this way, potentially erroneous results can be identified, and/or additional testing procedures can be recommended.

☑ CHECKPOINT 33-1

Assume that you are the clinical laboratory professional collecting a blood specimen from a patient with a suspected bleeding disorder. You noticed petechiae and several bruises on the patient's arm. What screening tests would likely have been ordered? What results of these tests would you expect (normal or abnormal) in this patient?

DISORDERS OF THE VASCULAR SYSTEM

Because the blood vessels are actively involved in hemostasis in a variety of ways (Chapter 31), a structural abnormality or damage either to the endothelial lining of blood vessels or the subendothelial structures can result from or lead to a variety of clinical manifestations and disease conditions. These disorders can be either inherited or acquired secondary to another condition. The hereditary vascular diseases are characterized by abnormal synthesis of subendothelial connective tissue or extracellular matrix components[4–10] (Table 33-1 ★). Either abnormal subendothelium or altered endothelial cells can cause acquired vascular disorders (Table 33-2 ★).

Symptoms seen in vascular disorders are for the most part superficial bleeding, such as easy bruising, petechiae, or lesions that

★ **TABLE 33-1** Characteristics of Inherited Disorders of the Connective Tissue

Disorder	Gene	Effect	Chromosome	Laboratory Findings
• Hereditary hemorrhagic telangiectasia (HHT)	Endoglin/*ENG* (HHT-1) *ACVRL-1* (HHT-2)	Arteriovenous malformations; ↑ VEGF	9q (HHT-1) 12q (HHT-2)	Usually normal
• Ehlers-Danlos syndromes	*COL1A1, COL1A2, COL3A1, COL5A1, COL5A2*	Fragility of blood vessels and tissues; joint hypermobility and instability	Multiple	BT can be increased
• Marfan syndrome	Fibrillin-1 (*FBN1*)	Overgrowth of long bones, arachnodactyly, joint hypermobility, decreased strength of blood vessels	15	Usually normal
• Osteogenesis imperfecta	Type I procollagen (*COLA1* and *A2*)	Patchy, defective bone matrix; brittle bones that fracture easily	2	Usually normal
• Pseudoxanthoma elasticum	*ABCC6*	Degeneration and calcification of elastic fibers	16	Usually normal

BT = bleeding time

mimic them but are actually not caused by bleeding. In the majority of patients with a primary vascular disorder, routine hemostasis testing is entirely normal, and the diagnosis of blood vessel disorders is most often made by exclusion. The platelet count, PT, and APTT are usually normal in a patient who has a history or physical examination suggesting a bleeding problem. The closure time, template bleeding time, and other platelet function testing are also usually normal but can be prolonged in some vascular disease states. Confirmation of a vascular disease usually requires histologic evaluation of the vessel wall or the underlying connective tissue or molecular analysis for the specific genetic mutation.

When platelets are normal in number and function, purpura are considered to be caused by damage to the blood vessels, a condition called **nonthrombocytopenic purpura**.

Hereditary Disorders of the Vascular System

Refer to Table 33-1 for a summary of hereditary disorders of the vascular system. These disorders are rare, and although bleeding is a common symptom, hemostasis tests are not informative in making the diagnosis. Some vascular disorders are associated with abnormal vessel wall integrity, but others manifest as vascular proliferative lesions. *Hemangiomas* are arteriovenous malformations resulting in direct connections between arteries and veins without intervening capillary beds. They are caused by either upregulation of growth-promoting factors and/or inhibition of apoptosis. Most hemangiomas appear in infancy and spontaneously involute over the next several years. In contrast, the telangiectasia lesions seen in hereditary hemorrhagic telangiectasia slowly progress over decades. A more complete description of these disorders is available in this chapter's Companion Resources.

Acquired Disorders of the Vascular System

Acquired disorders of the vascular system are seen quite often and are characterized by bruising and petechiae. Defects of either the vessel wall or the endothelial cells can be caused by (1) conditions that decrease the supportive connective tissue in blood vessel walls, (2) presence of abnormal proteins in the vascular tissues, (3) infections or allergic conditions, and (4) mechanical stress.[11] See Table 33-2 for acquired disorders of the vasculature.

Purpura Resulting from Decreased Connective Tissue

The diseases in this category result from a decreased amount of supportive connective tissue in the blood vessel walls.

Senile Purpura

Senile purpura are ecchymoses that appear with unrecognized or minor trauma in older individuals. With age, the extracellular matrix components of the skin degenerate, especially in body areas that have

★ **TABLE 33-2** Classification of Acquired Disorders of the Vascular System

Disorder	Examples
• Purpura resulting from decreased connective tissue	Senile purpura Cushing syndrome and glucocorticoid therapy Scurvy (vitamin C deficiency)
• Purpura associated with dysproteinemias	Paraproteins (cryoglobulinemia, cryofibrinogenemia) Amyloidosis
• Purpura resulting from vasculitis	Henoch-Schönlein purpura Infections Drugs
• Miscellaneous causes of purpura	Mechanical purpura Artificially induced purpura Easy bruising syndrome Purpura fulminans

been exposed to sunlight. This decreases the supportive collagen fibrils and allows small blood vessels, particularly capillaries, to burst and form bruises with minor pressure. The bruises do not heal easily and can last for months.

Cushing Syndrome and Glucocorticoid Therapy

Excess endogenous glucocorticosteroids (Cushing syndrome) or exogenous (therapeutic) glucocorticoids result in excessive breakdown of collagen, thin fragile skin, vessel wall fragility, and bruising. Small blood vessels can be mechanically broken and bleed into the skin.

Scurvy

Scurvy is a disease caused by a deficiency of vitamin C, which is needed for collagen synthesis. In its absence, collagen production is abnormal, resulting in vascular fragility and bleeding. Bleeding gums and bleeding around hair follicles on arms and legs are characteristic; ecchymoses and intramuscular hemorrhages are also seen. Treatment is oral vitamin C.

Purpura Associated with Dysproteinemias

The diseases in this category result from the presence of abnormal proteins in the vascular system.

Paraproteins

Paraproteins are normal or abnormal proteins appearing in large quantities as a result of a pathologic condition. They include cryoglobulins and cryofibrinogens, which are cold-insoluble immunoglobulins and abnormal fibrinogens. Monoclonal immunoglobulins are produced by monoclonal neoplastic plasma cells and occur in a variety of malignant conditions such as multiple myeloma, Waldenstrom's macroglobulinemia, and lymphoproliferative disorders (Chapter 28). Hemostatic symptoms include purpura, bleeding, and thrombosis. The mechanisms leading to these symptoms are varied and complex, including qualitative platelet defects, acquired inhibitors and deficiencies of coagulation factors, binding of calcium by paraprotein leading to interference with coagulation, and deposition of light chain protein in the vascular wall. Thrombocytopenia can be present because of the underlying disease and contributes to the bleeding.

Amyloidosis

Amyloidosis occurs as a primary disorder, as well as secondary to paraproteinemias, such as multiple myeloma. It is a condition in which deposits of amyloid (modified or misfolded proteins) form in the skin, perivascular tissue, and vessel walls. It leads to fragility of the vessels and bruising. Bleeding into visceral organs can also occur, and thrombosis is common.

Purpura Resulting from Vasculitis

Vasculitis is inflammation of the small blood vessels, often associated with infections. It can be caused by a direct attack on the vascular wall or when immune complexes (antigen–antibody complexes) attach either to the endothelial cells or the underlying subendothelial structures and activate complement.[12] The activation fragments of complement initiate several processes:

1. Neutrophils migrate to the area by chemotaxis and phagocytize the immune complexes. Enzymes, free oxygen radicals, and other substances are then released from the neutrophils and damage the vascular tissue.
2. Complement components C3a and C5a cause increased vascular permeability, resulting in vasodilation and edema.
3. The lytic complement cascade can be completed, resulting in damage to the vascular cell membranes.

Antigen–antibody complexes also cause aggregation of platelets and activation of factor XII (FXII) (Chapter 32), both of which contribute to thrombosis. Thrombi can occlude blood vessels causing **ischemia** of the supplied tissues and can result in tissue death or **necrosis**. Activated FXII results in the activation of kallikrein and the release of kinins from high-molecular-weight kininogens, which contribute to the vasodilation and edema associated with inflammation. The damaged vessels can rupture and produce localized purpura. Purpura caused by vasculitis is called *palpable purpura* because small nodules form at the site of the inflammation.

The cause of the vasculitis in most patients is never found. Some conditions that are associated with vasculitis are:

- Henoch-Schönlein purpura
- Infections
- Drugs

Details of these conditions can be found in this chapter's Companion Resources.

Miscellaneous Causes of Purpura

Other causes of purpura may or may not be related to abnormalities of the hemostatic system.

Mechanical Purpura

Increased pressure within the lumen of capillaries after intense exercise, coughing spasms, or epileptic seizures can cause petechial hemorrhages in the skin.

Artificially Induced Purpura

Artificially induced bruises can be self-inflicted (*factitious purpura*) or result from abuse by others. The cause is difficult to distinguish from a true physiologic (pathologic) process. Petechial purpura can be induced by placing negative pressure on the skin for a prolonged period as, for instance, sucking the air from a glass held to the mouth. Petechiometers used for the capillary fragility test were based on this principle.

Easy Bruising Syndrome

A benign condition called *easy bruising syndrome* (or *purpura simplex*) occurs more commonly in young women than men, and is thought to result from vascular fragility. Spontaneous small ecchymoses appear on the skin of the thighs or upper arms and have been called *devil's pinches*. The cause of this condition is not fully understood at the molecular level but is thought to result from hormonal effects on the blood vessel and/or its surrounding tissue.[13]

Purpura Fulminans

Purpura fulminans is a devastating type of purpura seen in newborns and others associated with abnormalities of certain clotting factors or their inhibitors and in some patients who are on therapy for thrombotic conditions (Chapter 35). In purpura fulminans, thrombi form in the small vessels supplying the skin and subcutaneous tissues. The vessels become occluded, the skin becomes necrotic, and the condition can rapidly lead to death.

CASE STUDY (continued from page 664)

Petechiae were noted on Mohammed's extremities. Blood work was ordered.

1. Which laboratory tests—hematology and hemostasis—would likely be ordered immediately and be most informative in interpreting the cause of the patient's bleeding?
2. Does the patient most likely have a disorder of primary or of secondary hemostasis? Why?

PLATELET DISORDERS

Platelets function in hemostasis to maintain the integrity of the blood vessels and to form the primary hemostatic plug in response to injury so that blood loss is minimized (Chapter 31). Bleeding from a cut finger stops when the primary hemostatic plug has sealed the openings in the injured vessels. Formation of the primary hemostatic plug requires an adequate number of normally functioning platelets. If platelet numbers are decreased (*thrombocytopenia*) or if platelet function is abnormal (*thrombocytopathy*), excessive bleeding can occur.

Platelet disorders are classified as quantitative (numerical) or qualitative (functional). *Quantitative disorders* are those in which the platelet count is either below (thrombocytopenia) or above (thrombocytosis) the reference interval. *Qualitative disorders* involve an abnormality of some aspect of platelet function.

Characteristic manifestations of the hemostatic defect seen in patients with platelet disorders are petechiae and excess bleeding from superficial areas of the body such as the skin and mucous membranes. **Epistaxis** (nose bleeds), excessive bleeding from cuts in the skin, and easy bruisability are commonly experienced. Other possible sites of bleeding are listed in Table 33-3 ★. The bleeding symptoms reflect the decreased ability of the platelets to form the primary hemostatic plug.

★ **TABLE 33-3** Types of Bleeding in Disorders of Primary Hemostasis

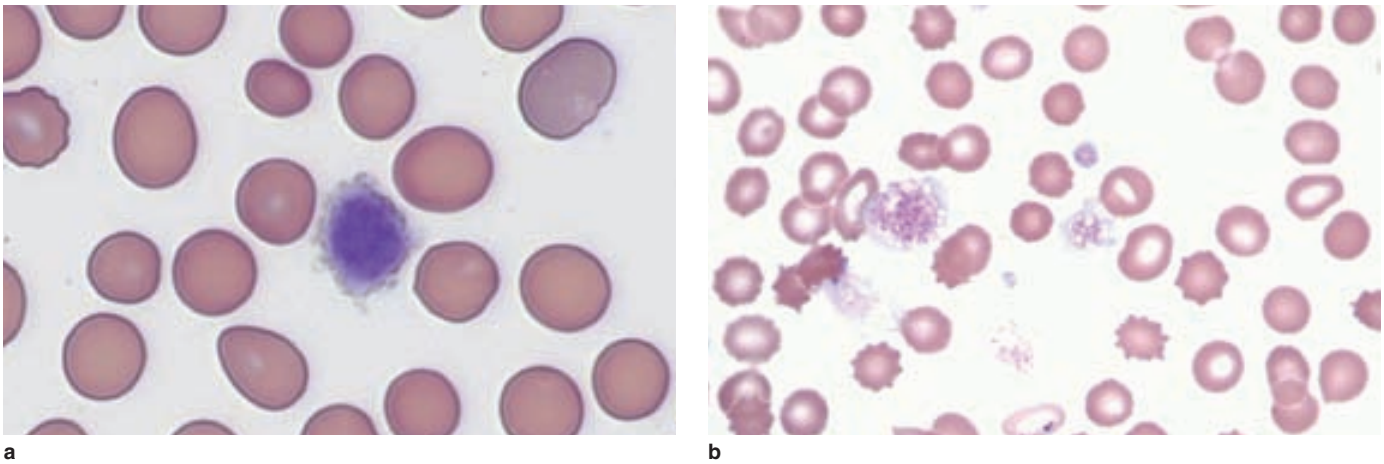
- Petechiae
- Ecchymoses
- Epistaxis
- Excessive bleeding from superficial wounds
- Bleeding into the retina
- Gastrointestinal bleeding
- Bleeding in the urinary tract
- Hypermenorrhea
- Gingival bleeding (gums)
- Increased bleeding after tooth extraction
- Intracranial bleeding

Manifestations of increased platelet counts generally reflect the underlying problem that caused the thrombocytosis; occasionally, there is increased likelihood of thrombosis (or rarely, hemorrhage).

Quantitative Platelet Disorders

Quantitative abnormalities of platelets include thrombocytopenia or thrombocytosis. A reference interval of 150–400 $\times$ 10^9/L (Table C, inside front cover) is similar to that established by most laboratories. Platelets are usually counted by automated instruments, although some laboratories use manual counting methods as a backup. Methods of counting platelets and of correlating the automated platelet count with the number of platelets found on a peripheral blood smear are described in Chapter 9.

The blood smear can also include the presence of morphologically abnormal forms of platelets, such as platelets larger (macrothrombocytes) or smaller (microthrombocytes) than normal, those with decreased or absent granules (hypogranular or agranular), or a combination of variables. The clinical laboratory professional should recognize and report platelet variants. Some laboratories use the term *giant platelet* to describe all abnormally large platelets (Figure 33-2 ■).



a b

■ **FIGURE 33-2** **(a)** Large granular platelet. **(b)** Increased numbers of platelets, some of which have normal morphology, some of which are hypogranular, and several giant platelets (peripheral blood, Wright-Giemsa stain, 1000× magnification).

★ **TABLE 33-4** Significant Laboratory Tests in Defects of Primary Hemostasis

	Screening Tests			
	Platelet Count	**Prothrombin Time**	**APTT**	**Template BT**
Vascular disorders	Normal	Normal	Normal	Normal or abnormal
Thrombocytopenia	Decreased	Normal	Normal	Abnormal
Platelet dysfunction	Usually normal	Normal	Normal	Normal or abnormal

☑ CHECKPOINT 33-2

If you observed an average of 14 platelets per high power field on a peripheral blood smear prepared from the needle tip and your laboratory allowed correlation between the direct instrument count and the blood smear estimate of 20%, what range would you expect the instrument count to be? Is this platelet estimate within an acceptable reference range?

Thrombocytopenia

The lower limit of the reference interval for platelets is approximately 150×10^9/L. The clinical significance of borderline thrombocytopenia ($100–150 \times 10^9$/L) is unclear. However, severe thrombocytopenia is usually associated with clear clinical complications.[14] Clinical symptoms are usually not seen unless the count falls below 100×10^9/L. When it is below 50×10^9/L, the severity of clinical manifestations can to some degree parallel the platelet count. Possible symptoms include petechiae, menorrhagia, or spontaneous bruising with little or no trauma. The possibility of severe and spontaneous bleeding in otherwise hemostatically healthy individuals usually does not occur until the platelet count is below 10×10^9/L.[14] Fatal bleeding into the central nervous system as well as spontaneous bleeding from mucous membranes (e.g., the gastrointestinal tract, the genitourinary tract, and the nose) can occur. Replacement therapy (platelet transfusions) is generally not indicated (in the absence of overt bleeding) unless the platelet count falls to this level. The extent of symptoms at all platelet levels varies from patient to patient and can be affected by medications, the blood vessel status, impaired platelet function, fever, sepsis, or other concurrent disease. The number of circulating platelets is usually significantly in excess of what is required for normal hemostasis.

Laboratory tests for coagulation factors (PT and APTT) and for fibrinolysis are unaffected by thrombocytopenia. Table 33-4 ★ compares typical laboratory screening test results in thrombocytopenia, disorders of abnormal platelet function, and vascular disorders.

Thrombocytopenia, the most common cause of excess or abnormal bleeding, is not a disease but a symptom of an underlying condition, as is anemia. Finding a decreased platelet count with or without abnormalities of other hematologic parameters suggests the need to search for the underlying cause so that the condition can be appropriately treated. A decreased platelet count can be the primary feature or a secondary manifestation of several conditions. This text classifies thrombocytopenia into five major categories based on pathophysiology (Table 33-5 ★).

Increased Destruction

The most common cause of thrombocytopenia is increased destruction of platelets, resulting in a decreased platelet life span after they have been released into the peripheral blood. A normal bone marrow attempts to compensate for the decreased platelet numbers in the peripheral blood by increasing production. Consequently, the number of bone marrow megakaryocytes is normal to increased. However, the platelets may be eliminated from the circulation faster than the bone marrow can produce them, resulting in thrombocytopenia (Figure 33-3 ■). Because of the unique mechanism of thrombopoietin (TPO) regulation of platelet production, plasma levels of TPO are not increased above the normal range in most patients with thrombocytopenia due to increased destruction[15] (Chapter 9). Causes of increased destruction include both immune-mediated and nonimmune destruction of platelets (Table 33-6 ★).

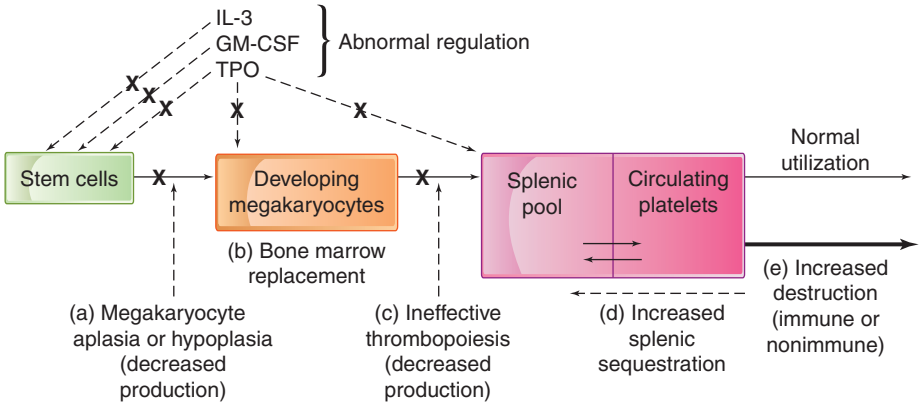
Immune-Mediated Destruction. Immune platelet destruction is caused by antibodies and is analogous to the destruction of erythrocytes in immune hemolytic anemias (Chapter 19). Platelets become coated or "sensitized" with antibody, and mononuclear phagocytes, primarily in the spleen, destroy them. Monocytes and macrophages possess Fc receptors (FcγR) by which they recognize immunoglobulin-coated platelets (Figure 33-4 ■). Liver macrophages can remove platelets coated with large amounts of antibody.

★ **TABLE 33-5** Classification of Quantitative Platelet Abnormalities

Thrombocytopenia
- Increased destruction
  - Immune
  - Nonimmune
- Decreased production
  - Megakaryocyte hypoplasia
  - Replacement of normal marrow
  - Ineffective thrombopoiesis
  - Inherited disorders
- Increased splenic sequestration
- Dilutional thrombocytopenia
- Conditions with multiple mechanisms of thrombocytopenia

Thrombocytosis
- Primary thrombocytosis
- Secondary thrombocytosis
- Transient thrombocytosis

Artifacts in the quantitative measurement of platelets



■ **FIGURE 33-3** Schematic drawing showing various causes of thrombocytopenia.
IL-3 = interleukin-3; GM-CSF = granulocyte macrophage colony stimulating factor; TPO = thrombopoietin; X = abnormal or absent

Antibodies attach to platelets either by their Fab regions, binding to specific epitopes on GPIIb/IIIa or Ib/IX or by nonspecific attachment of immune complexes to platelet Fc receptors (Figure 33-4). The antibodies can be IgG, IgA, or, rarely, IgM, and complement can become activated. The level to which the platelet count becomes decreased depends on the concentration and activity of the antibody, the function of the macrophage Fc and complement receptors, and the ability of the bone marrow to increase platelet production to compensate for the increased loss.

Unlike the immune hemolytic anemias, routine laboratory tests designed to quantitate the amount of immunoglobulin on platelets of thrombocytopenic patients are not available. Although several laboratory assays have been developed to measure the immunoglobulin on the surface (**p**latelet-**a**ssociated **i**mmuno**g**lobulin [PAIg]), they lack both sensitivity and specificity, and thus are rarely used diagnostically.[14]

★ **TABLE 33-6** Classification of Thrombocytopenia Caused by Increased Destruction

Immune mechanisms of destruction
- Immune thrombocytopenic purpura
  - Acute
  - Chronic
  - Transplacental
- Alloimmune thrombocytopenia
  - Neonatal alloimmune thrombocytopenia (NAIT)
  - Post-transfusion purpura
- Drugs
  - Quinidine/quinine
  - Heparin
- Other diseases

Nonimmune mechanisms of destruction
- Disseminated intravascular coagulation
- Thrombotic thrombocytopenic purpura
- Hemolytic uremic syndrome
- Mechanical destruction by artificial heart valves

In immune-mediated thrombocytopenias, the thrombocytopenia is typically the only hematologic abnormality present, so bone marrow examination is rarely needed.[14] If performed, it can evaluate megakaryocyte number and morphology and rule out other causes of thrombocytopenia. Megakaryocytes (MKs) are increased, often markedly, reflecting stimulation by TPO to increase platelet production. In cases of severe thrombocytopenia, the marrow can increase production up to five times normal. The ploidy of MKs also can be increased, resulting in a larger cytoplasmic mass and an increased number of platelets produced per MK (Chapter 9). In autoimmune thrombocytopenia (see the next section), some patients' antibodies appear to react with marrow MKs and can interfere with platelet production. Although MK number and morphology appear normal when viewed on a Romanowsky-stained smear by light microscopy, they may show extensive damage when viewed by electron microscopy.[15]

Autoimmune Thrombocytopenia. The most common form of thrombocytopenia is **immune thrombocytopenia (ITP)** (formerly called *immune thrombocytopenic purpura*).[16] It can occur as either a primary (idiopathic) disorder, or secondary to a number of associated diseases. Historically, ITP has been described as an autoimmune disorder in which autoreactive antibodies bind to platelet glycoproteins, shortening the platelet life span. Studies over the past 20 years have indicated that the immune dysregulation is more complex. In addition to abnormal, autoreactive B lymphocytes (autoantibody production), immune abnormalities can include abnormal regulatory T cells (T_{Reg}) that normally function to contain autoimmune reactions, and loss of T-cell tolerance against platelet antigens with the accumulation of autoreactive cytotoxic T cells.[15] The clinical presentation of affected individuals can vary from an asymptomatic thrombocytopenia to severe mucosal bleeding.

Because of the difficulty in identifying platelet-specific antibodies, the diagnosis of ITP has been and continues to be made by exclusion (i.e., ruling out all other causes of thrombocytopenia). The diagnosis is based on the patient's history and physical examination, complete blood counts, and examination of the blood smear. Characteristically, patients present with an isolated thrombocytopenia with a peripheral blood platelet count of $<100 \times 10^9$/L.[16] Except

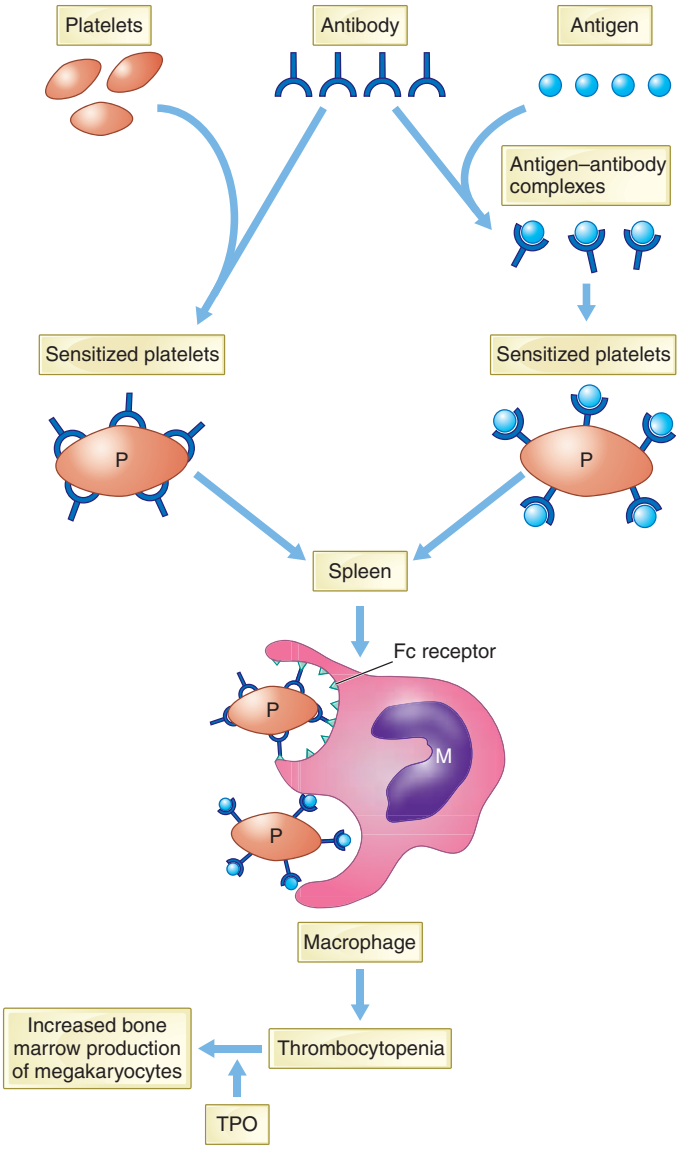


FIGURE 33-4 Mechanisms of thrombocytopenia caused by immune destruction of platelets. In immune thrombocytopenia, Fc receptors of the splenic macrophages (M) recognize antibody-sensitized platelets and eliminate them from the circulation. The bone marrow is stimulated by thrombopoietin (TPO) and produces increased numbers of megakaryocytes. Thrombocytopenia develops if immune destruction exceeds the compensatory capacity of the marrow. The antibody can also injure megakaryocytes.

for thrombocytopenia, the findings on a peripheral blood smear are within normal intervals for the age of the patient unless an underlying condition exists. Other tests are usually not indicated.

Historically, ITP was defined as either acute (resolution of the thrombocytopenia within 6 months) or chronic (thrombocytopenia persisting >6 months). In 2009, the International Working Group for ITP consensus report recommended dividing the disorder into three phases: newly diagnosed ITP (duration up to 3 months), persistent ITP (duration 3 months to 1 year), and chronic ITP (cITP) (duration >1 year).[16]

The clinical features of ITP in children and adults are different (Table 33-7 ★). During the time period encompassing newly diagnosed and persistent ITP, 85% of children have a spontaneous remission, whereas only 15% of adults spontaneously remit during the same time period. ITP occurs most often in children 5–6 years of age, shows no gender preference, and often follows a viral infection by 1–3 weeks. Nonspecific upper respiratory infections, chickenpox, rubella, rubeola, cytomegalovirus, and viral hepatitis are some diseases that have been associated with ITP in children.

The platelet count is often $< 20 \times 10^9$/L. Bleeding manifestations vary from none to mild to moderate and can include the abrupt onset of easy bruising, petechiae on the extremities, and bleeding from mucous membranes. Life-threating bleeding is rare (<1% of cases) with spontaneous bleeding into the central nervous system being the most dreaded consequence.[15] There can be an increase in the number of lymphocytes or mild eosinophilia in some cases, most likely related to the preceding infection. Bone marrow examination is typically not needed.

Most children experience a spontaneous remission within 2–6 weeks of the onset of the illness regardless of treatment,[15] and the disease does not recur in these patients. However, therapy may be useful in reducing the period of severe thrombocytopenia; 80–90% of children will remit by 12 months. Only 5–10% develop cITP.

☑ CHECKPOINT 33-3

How many platelets per 1000× field would you expect to observe on the peripheral smear of a patient with acute ITP?

ITP in adults differs from ITP in children in several aspects, including typical patient age, female predominance, insidious onset, and initial platelet count (Table 33-7). ITP in adults is most commonly a chronic disorder and rarely has a spontaneous resolution.

The diagnosis is made based on the patient's medical history, physical examination, peripheral blood counts, and blood smear examination. Of adult patients with ITP, 30–40% are asymptomatic and are diagnosed only by the incidental finding of a low platelet count.[15] Bleeding symptoms are rare unless the thrombocytopenia is severe ($<30 \times 10^9$/L). The erythrocyte and leukocyte morphology are usually normal; thrombocytopenia is the only significant finding. Bone marrow examination is not recommended unless it is necessary to rule out other diseases with similar presentation, such as acute leukemia, aplastic anemia, or myelodysplastic syndrome. Human immunodeficiency virus (HIV) and hepatitis C virus (HCV) testing should be routinely performed on adults with newly diagnosed thrombocytopenia.[17]

Antiplatelet autoantibodies can be identified in 58–80% of patients with chronic ITP. Most are directed toward epitopes on either GPIIb/IIIa or GPIb/IX.[17] Although the numbers of megakaryocytes in the bone marrow are normal or increased, platelet production is generally decreased to low normal, probably because of an effect of antiplatelet antibodies or cytotoxic T cells on the marrow megakaryocytes.[15]

Bleeding manifestations rarely occur until platelet counts fall below $20–30 \times 10^9$/L; thus, initial treatment for patients (both adults and children) with ITP may simply involve preventing trauma to reduce the risk of bleeding and observation.[18] Because of the high spontaneous remission rate in children, they usually require no specific

★ **TABLE 33-7** Comparative Features of Childhood and Adult ITP

Feature	Childhood	Adult
Peak age incidence	5–6 years	20–40 years
Platelet count, initial	$<20 \times 10^9$/L	$30–80 \times 10^9$/L
Onset of symptoms	Abrupt	Insidious
Type of bleeding	Petechiae and superficial	Petechiae and superficial
Antecedent infection	Common 1–3 weeks prior	Unusual
Gender predilection	None	Females 3:1
Eosinophilia	Common	Rare
Lymphocytosis	Common	Rare
Hemorrhagic bullae in mouth	Present in severe cases	Usually absent
Duration	2–6 weeks	Months or years (lifetime)
Spontaneous remissions	Up to 95% of cases	Rare (<15% of cases); course of disease fluctuates
Therapy	Corticosteroids, anti-D, IVIg	Corticosteroids, splenectomy, Rituximab

IVIg = intravenous immunoglobulin

treatment. Most adults eventually require treatment because of the low rate of spontaneous remission. The goal of treating patients, if needed, is to raise the platelet count to a level that supports adequate hemostasis as rapidly as possible while preventing life-threatening bleeding. The goal of ITP treatment is thus to reach a safe platelet count, not a normal platelet count. The American Society of Hematology recently published guidelines for the management of ITP,[18] recommending a platelet count $<30 \times 10^9$/L as the threshold for considering treatment unless the patient is bleeding.

Initial treatment approaches usually involve corticosteroids, intravenous immunoglobulin (IVIg) or anti-D. Although 60–70% of adults achieve an initial response with corticosteroids, <50% sustain the response at 6 months. Both high-dose IVIg and anti-D are thought to work by blocking the Fcγ receptors on macrophages in the spleen and liver and are generally effective only in nonsplenectomized patients. The use of anti-D is an option for Rh-positive individuals because anti-D-coated patient RBCs compete for Fcγ receptor occupancy on macrophages. Although effective at increasing the platelet count temporarily, it is not a long-term therapeutic option for cITP. If patients fail to respond to first-line therapies, the choice of subsequent therapy is controversial. Splenectomy has been used for >80 years and is successful at raising the platelet count. The spleen is not only the site of the removal of the majority of sensitized platelets but also a major site for antibody production. However, splenectomized patients are at increased risk of infection with encapsulated bacteria (*Streptococcus*, *Neisseria*, *Haemophilus*); thus, immunization against encapsulated bacteria at least 2 weeks prior to surgery is recommended.[17] Recently rituximab, an anti-CD20 antibody, has been used with some success to destroy B lymphocytes, which are involved in autoantibody production. Currently, thrombopoietin receptor agonists are being evaluated to increase platelet counts to a hemostatically safe level.[17] For patients who are refractory to these therapies, the most aggressive treatments include chemotherapeutic agents such as vincristine or vinblastine or immunosuppressive therapies such as cyclosporin. In general, replacement therapy with platelet transfusions should be used only in life-threatening situations.

The clinical course of ITP in some patients consists of alternate periods of remission within 6 months of the onset followed by relapse after at least 3 months. This is referred to as an *intermittent* form of the disease.

Transplacental (or neonatal) ITP is an immune form of thrombocytopenia that occurs in newborn infants of mothers with ITP. It is estimated to affect 1–2 of every 10,000 pregnancies.[19] Approximately 15– 65% of newborns of mothers with ITP have thrombocytopenia at birth; of them 6–70% will have severe thrombocytopenia with platelet counts of $<50 \times 10^9$/L. The maternal platelet count does not correlate with that of the fetus, so there is no way to predict which neonates will be at risk. The major hazard is intracranial bleeding during delivery. Thrombocytopenia lasts until the maternal antibody is cleared from the newborn's system, an average of 3–4 weeks postdelivery.[19]

ITP can occur secondarily to a number of underlying disorders, including chronic HIV and HCV infections; infection with *Helicobacter pylori* and the Epstein Barr virus; lymphoproliferative disorders; and other autoimmune disorders such as systemic lupus erythematosus (SLE) and antiphospholipid antibody syndrome (APLS). Thrombocytopenia occurs in more than one-third of patients with bacterial septicemia, although the platelet count is usually not $<50 \times 10^9$/L and resolves when the infection is treated.

Alloimmune Thrombocytopenias. Immune platelet destruction can be caused by alloantibodies stimulated by foreign antigens during pregnancy or after blood transfusions. **Neonatal alloimmune thrombocytopenia (NAIT)** is similar to hemolytic disease of the fetus and newborn except that the antibodies are directed toward platelet antigens rather than erythrocyte antigens, and it commonly occurs in a first pregnancy. Platelet destruction in the newborn is the result of maternal alloantibodies formed against a platelet-specific antigen the baby inherited from the father and that the mother lacks.[17] Maternal IgG antiplatelet antibodies cross the placenta, destroying the infant's platelets.

The target of the alloantibodies are epitopes on platelet glycoproteins, usually resulting from single nucleotide polymorphisms (SNPs).[17] The majority of alloantigens are associated with GPIIIa, although other glycoproteins have been implicated as well. The most frequently encountered platelet antigen causing NAIT is HPA-1a (PLA1) (>80% of cases of NAIT).[17] Approximately 3% of the Caucasian population is homozygous for HPA-1b (PLA2) and will be

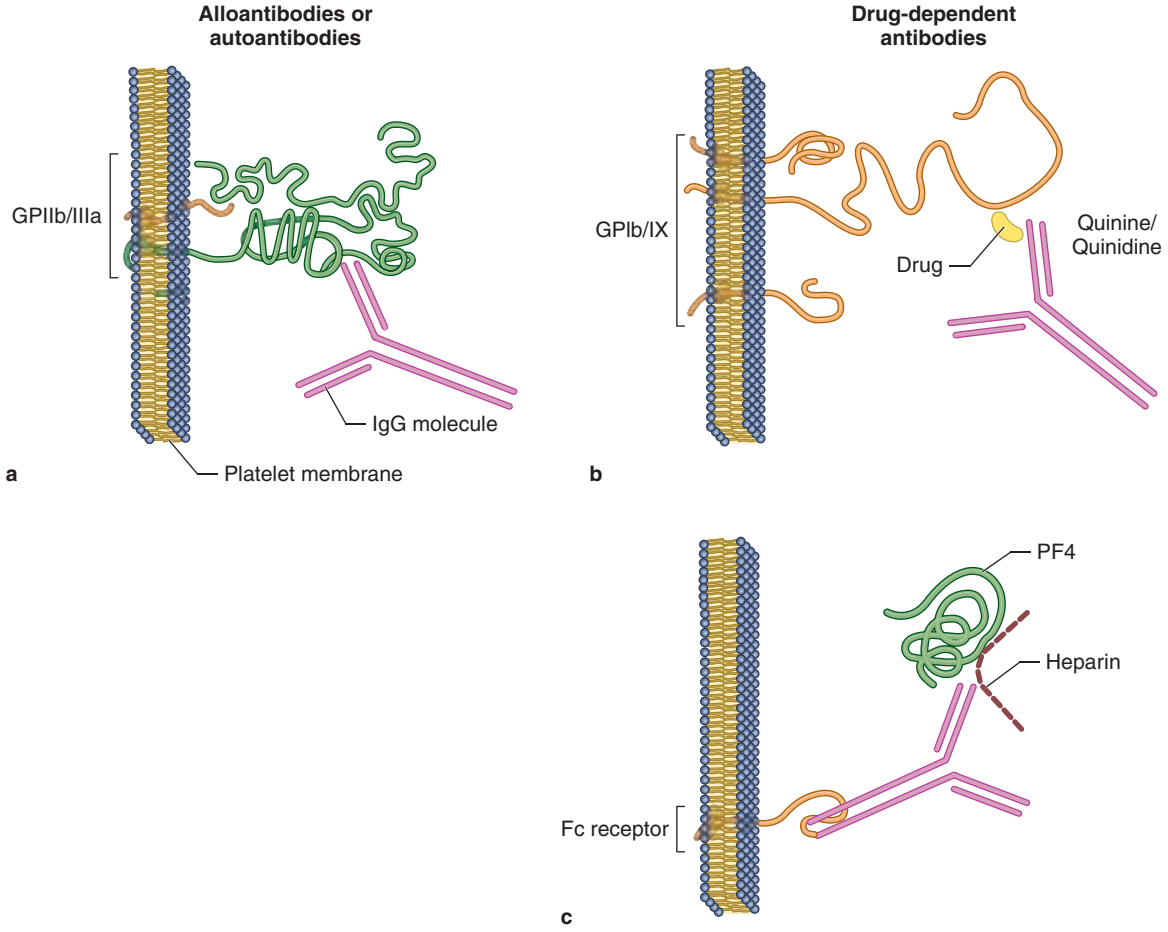
immunized if exposed to HPA-1a. Figure 33-5a ■ shows a proposed mechanism of attaching alloimmune or autoimmune antibodies to the platelet surface.

Symptoms appear at or shortly after birth and are self-limited. Bleeding occurs in ~20% of neonates with NAIT.[17] Bleeding symptoms can range from mild to severe and life-threatening. The overall mortality for infants with severe thrombocytopenia is ~10%. Intracranial bleeding, sometimes a result of birth trauma, can result in severe lifelong neurologic deficits in infants who survive. Thus, it is important to recognize NAIT and treat it with IVIg or transfusion with HPA-1a negative platelets as soon as possible.[17]

Post-Transfusion Purpura. Rarely, patients develop severe thrombocytopenia 5–10 days after blood transfusion (post-transfusion purpura [PTP]). Approximately 90% of affected patients are women. Most are homozygous for the HPA-1b polymorphism and have produced an alloantibody against the HPA-1a antigen; however, other platelet-specific antigens have been implicated as well.[15] The mechanism of alloantibody destruction of autologous platelets negative for the antigen is controversial, and more than one mechanism may be operative.[13] One hypothesis is that PTP is triggered by binding antigen–antibody immune complexes to autologous platelets, resulting in their clearance by macrophages. Symptoms such as bleeding from mucous membranes and purpura begin abruptly and usually last 2–6 weeks. However, prolonged severe thrombocytopenia can result in life-threatening bleeding, necessitating treatment with IVIg with or without corticosteroids.[15]

Drug-Induced Thrombocytopenia. Drug-induced thrombocytopenia is relatively common, and drugs can induce it through a variety of mechanisms. Some drugs inhibit platelet production either selectively or by a generalized suppression of marrow hematopoiesis. Other drugs cause thrombocytopenia by promoting platelet destruction, generally by drug-dependent antibodies.[20,21] A list of the drugs that have been identified as causing thrombocytopenia is accessible at http://w3.ouhsc.edu/platelets (accessed January 11, 2014).

More than 200 drugs have been reported to cause thrombocytopenia. Drugs more commonly associated with immunologic thrombocytopenia are quinidine, quinine, vancomycin, gold salts, sulfonamides and derivatives, and heparin. In some cases, the



■ **FIGURE 33-5** Variations in antibody binding to platelets. **(a)** Platelet autoantibodies or alloantibodies bind to epitopes on GPIIb/IIIa, GPIb/IX, most frequently, or to other glycoproteins resulting in ITP. **(b)** Quinine-/quinidine-dependent antibodies bind to a complex of drug and glycoprotein, usually either GPIb/IX or IIb/IIIa. **(c)** Heparin binds platelet factor 4, and the heparin/PF4 complex attaches to IgG antiheparin antibodies via the Fab combining site. The Fc portion of the antibody binds to platelet FcγIIa receptors.

offending agent is a metabolite of the drug formed in vivo rather than the original form of the drug.

Symptoms of excess bleeding appear suddenly and can be severe. The platelet count can be very low, often $<20 \times 10^9$/L. Bleeding manifestations include petechiae, purpura, and, occasionally, intracranial hemorrhage. On first exposure, symptoms appear 1–2 weeks after taking the drug. After the patient has been sensitized, symptoms can occur within hours after taking a dose. Usually the only necessary treatment is withdrawal of the offending drug. The platelet count typically begins to rise within a few days and symptoms subside, although occasionally several weeks are required.[21] The exception is the thrombocytopenia induced by α-methyldopa and gold, a condition in which drug-induced autoantibodies bind to platelets even in the absence of the drug. The mechanism by which drugs can induce the formation of platelet-specific autoantibodies is unclear.

Three pathways have been proposed to explain drug-induced immune-mediated platelet destruction.[20] The first mechanism, the *hapten theory*, proposes that the drug binds covalently with platelet membrane glycoproteins to form a drug–platelet antigenic complex, and the drug acts as a hapten. A drug-dependent antibody that recognizes and binds to this complex is formed. However, few studies support the hapten model of antibody formation except for perhaps the thrombocytopenia seen with large doses of penicillin. The second model was the *innocent bystander mechanism* in which the drug binds to a plasma protein and elicits an antibody response. Antibody binding to the drug-protein complex forms an immune complex that nonspecifically binds to circulating platelet Fc receptors (Figure 33-5c). Again, with the exception of some heparin-induced thrombocytopenias, most drugs do not appear to work via this mechanism.

The most common mechanism is the formation of drug-dependent antibodies against epitopes created by the association of the drug with proteins on the platelet surface. The association can be a specific ligand–receptor interaction, often with GPIIb/IIIa molecules, or a nonspecific noncovalent interaction. Some drugs can create neoepitopes for antibody formation by interacting with the platelet surface glycoproteins. Antibodies to quinidine and quinine bind to either drug/GPIb/IX or drug/GPIIb/IIIa complexes on the platelet surface by their Fab portions (Figure 33-5b).

The mechanism of platelet destruction is similar to that described previously: antibodies bind to the platelet, and macrophages remove the sensitized platelet. Drug-induced thrombocytopenias occasionally are caused by inhibition of megakaryocyte proliferation and platelet production as well as destruction of platelets in the circulation.

Heparin can cause thrombocytopenia via two mechanisms, one immune mediated **(heparin-induced thrombocytopenia [HIT])** and the other nonimmune mediated **(heparin-associated thrombocytopenia [HAT])**. Heparin is used to prevent or treat thrombosis (Chapter 35). In some patients, heparin causes a direct platelet activation effect, a nonimmune-mediated thrombocytopenia, resulting in HAT. HIT refers to the situation characterized by immune-mediated destruction of platelets because of heparin-dependent, platelet-activating IgG antibodies that recognize complexes of platelet factor 4 (PF-4) bound to heparin. PF-4 is released from platelet αG during platelet activation, binds to heparin in the circulation, and antibodies are produced to the heparin/PF-4 complex. The antibody/heparin/PF-4 complex then attaches to the platelet surface via the platelet FcγIIa receptors, resulting in increased platelet clearance and thrombocytopenia. Occasionally, the antibody-heparin-PF4 complexes can induce platelet activation (Figure 33-5c). The most serious complication of HIT is the activation of the clotting system and thrombosis (Chapter 35). Thrombocytopenia develops in approximately 3% of patients who receive heparin, and thrombosis occurs in 2–33% of those.[22] For this reason, patients receiving heparin therapy should be monitored with daily platelet counts. For a complete discussion of the role of HIT in inducing thrombosis, see Chapter 35.

Miscellaneous Thrombocytopenias. Thrombocytopenia occurs as a secondary feature in many diseases. Although a variety of mechanisms can contribute to thrombocytopenia in these disease states, there appears to be an immune element in collagen diseases, other autoimmune disorders including systemic lupus erythematosus and rheumatoid arthritis, lymphoproliferative disorders such as Hodgkin disease and chronic lymphocytic leukemia, and solid tumors (see secondary ITP in the "Autoimmune Thrombocytopenia" section).

☑ CHECKPOINT 33-4

A 6-year-old boy was brought to his pediatrician because the mother noticed small pinkish spots on his legs. Examination discovered several bruises on his arms and legs. Laboratory tests were ordered. His platelet count was 20×10^9/L, and the PT and APTT were within normal limits. The complete blood count (CBC) was normal except for the low platelet count. The boy had no previous history of bleeding. The mother said she had noted the spots after he was given the hepatitis vaccine. What is the most probable type of thrombocytopenia? Should other coagulation tests be performed at this time?

Nonimmune Destruction. Increased destruction of platelets can occur by processes other than immune-related mechanisms. Platelets can be activated and consumed by aggregation within the circulation. **Disseminated intravascular coagulation (DIC)** is a disorder in which fibrin formation within the blood vessels is associated with platelet activation and consumption (Chapters 34, 35). **Thrombotic thrombocytopenic purpura (TTP)** and **hemolytic uremic syndrome (HUS)** are conditions in which platelets are activated without generalized activation of the coagulation cascade. These are discussed in Chapter 35 with other hypercoagulable disorders and briefly in Chapter 20 with other causes of hemolytic anemias (erythrocyte destruction is also a classic feature of these disorders).

Mechanical destruction of platelets occurs in patients with artificial heart valves or vascular grafts. Platelets are destroyed by adhering to the artificial prosthesis or by mechanical rupture as the valves open and close against them.

Decreased Production

A second category of disorders causing thrombocytopenia is characterized by failure of the bone marrow to deliver adequate numbers of platelets to the peripheral blood. The bone marrow function in these disorders is abnormal, and thrombocytopenia develops secondarily.

Examination of bone marrow smears and sections aids in identifying the primary condition. The decreased platelet count occurs because of one of the following: (1) megakaryocyte hypoplasia in the bone marrow, (2) ineffective thrombopoiesis, or (3) a hereditary condition that affects the ability of the bone marrow to support megakaryocyte growth (Table 33-8 ★).

Megakaryocyte Hypoplasia Syndromes. Hypoplasia of megakaryocytes (Figure 33-3) can result from either decreased proliferation of megakaryocytes in the bone marrow or replacement of the bone marrow by neoplastic disease, fibrosis, or granulomatous inflammation. All three myeloid cell lineages can be affected or only the megakaryocytes.

Decreased Megakaryocyte Proliferation The most frequent cause of marrow hypoplasia is drug or radiation therapy for malignant disease. Chemotherapeutic agents and radiation produce generalized marrow suppression affecting all three lineages. The marrow usually regenerates shortly after the therapy is stopped, but the megakaryocytes are often the last lineage to recover.

Aplastic Anemia Megakaryocytes are decreased in aplastic anemia, a bone marrow disease characterized by pancytopenia and bone marrow hypoplasia. Acquired and constitutional aplastic anemias are discussed in Chapter 16. In acquired aplastic anemia, a decreased platelet count can appear before hypoplasia of other cell lineages and can be the last lineage to return to normal after recovery. Platelet size (mean platelet volume [MPV]) is normal in aplastic conditions in contrast to the increased MPV seen in immune-mediated thrombocytopenia. Platelets can, however, demonstrate an increased variation in size as indicated by the platelet distribution width (PDW) on electronic instruments. TPO levels are increased in patients with aplastic anemia in contrast to normal levels in immune-mediated platelet destruction.

Several hereditary disorders result in a congenital aplastic anemia. **Fanconi anemia (FA)** (Chapter 16) is a congenital disorder characterized by chromosomal instability, defective DNA repair mechanisms, and progressive bone marrow hypoplasia, eventually resulting in aplastic anemia in ~90% of affected patients. At least 15 different genes have been associated with the development of FA.[23]

Congenital amegakaryocytic thrombocytopenia (CAMT) presents with isolated hypomegakaryocytic thrombocytopenia during the first years of life but eventually converts into bone marrow failure and aplastic anemia, usually between the ages of 3–20.[24] The molecular basis for most patients has been identified as a mutation within the gene for the thrombopoietin receptor, TPO-R (*MPL*). The evolution from an isolated thrombocytopenia to complete marrow hypoplasia underscores the importance of TPO and its receptor in the maintenance of hematopoietic stem cells.

Congenital thrombocytopenia with radioulnar synostosis (CTRUS) is also a congenital disorder presenting as an isolated amegakaryocytic thrombocytopenia that evolves into aplastic anemia.[24] Most patients have a mutation within the *HOXA11* gene, resulting in a truncated HOXA11 protein, an important regulatory protein for development of both hemopoietic and bone tissue. The abnormal bone morphology usually presents as a union of the ulna and radius by osseous tissue and sometimes other skeletal abnormalities.

Thrombocytopenia with absent radii (TAR) is an inherited condition characterized by isolated hypoplasia of the megakaryocyte lineage, thrombocytopenia, and bilateral radial aplasia. Other skeletal malformations occasionally can be seen. The thrombocytopenia is usually severe only during the first years of life with a gradual improvement in platelet count to within normal values in adulthood. No mutations within the genes for TPO or TPO-R have been identified, and the defect is assumed to be an abnormality in the TPO/TPO-R signaling pathway.[24]

X-linked thrombocytopenia (XLT) is an X-linked inherited thrombocytopenia that can occur with or without anemia as the result of mutations in the gene for the transcription factor GATA-1, which plays a key role in both megakaryocytopoiesis and erythropoiesis. Depending on the exact mutation inherited, patients can either present with isolated thrombocytopenia (XLT), or a combined disorder of **X-linked thrombocytopenia with thalassemia (XLTT)**.

Replacement of Normal Marrow. Abnormal cells, malignant or nonmalignant, can replace normal marrow, decreasing the number of megakaryocytes (as well as the precursor cells for the other marrow lineages). Some myelodysplastic syndromes have decreased platelets as part of their pathology (Chapter 25). The bone marrow can have normal or increased numbers of megakaryocytes, many of which are micromegakaryocytes

★ **TABLE 33-8** Causes of Decreased Platelet Production

Hypoplasia of megakaryocytes

- Decreased megakaryocyte proliferation
  - Chemotherapy and radiation therapy for malignant disease
  - Aplastic anemia
    - Acquired
    - Congenital/Hereditary
      - Fanconi anemia
      - Congenital amegakaryocytic thrombocytopenia (CAMT)
      - Congenital thrombocytopenia with radioulnar synostosis (CTRUS)
  - Isolated megakaryocyte hypoplasia
    - Thrombocytopenia with absent radii (TAR)
  - X-linked thrombocytopenia (XLT)
    - X-linked thrombocytopenia with thalassemia (XLTT)
- Replacement of normal marrow
  - Leukemias and lymphomas
  - Myelodysplastic syndromes
  - Other neoplastic diseases at times
  - Fibrosis or granulomatous inflammation

Ineffective thrombopoiesis

- Megaloblastic anemia

Hereditary thrombocytopenia

- Wiskott-Aldrich syndrome/X-linked thrombocytopenia
- Bernard-Soulier syndrome
- May-Hegglin anomaly

and dysplastic forms. Peripheral blood platelets also can have abnormal morphology and abnormal function as indicated in platelet aggregation assays. Marrow replacement by solid tumors or by fibrous tissue, as in chronic idiopathic myelofibrosis, sometimes results in thrombocytopenia, although thrombocytosis is more common. Abnormal platelet morphology, such as large and/or hypogranular forms, can be present.

Ineffective Thrombopoiesis. Megaloblastic anemias caused by a deficiency of vitamin B_{12} or folic acid are characterized by ineffective thrombopoiesis as well as ineffective erythropoiesis and granulopoiesis (Figure 33-3) (Chapter 15). Pancytopenia is often seen in these diseases. The bone marrow contains normal or increased numbers of megakaryocytes, but the number of platelets entering the peripheral blood is decreased.

Hereditary Thrombocytopenia. Wiskott-Aldrich syndrome, Bernard-Soulier syndrome, and May-Hegglin anomaly are inherited disorders in which platelet production is decreased. These disorders also demonstrate other abnormalities.

The **Wiskott-Aldrich Syndrome (WAS)**, an X-linked disorder, is characterized by very small platelets, thrombocytopenia, and severe immune dysregulation (eczema and infections) resulting from a progressive decline in T lymphocyte number and function.[23] The *WAS* gene codes for a protein expressed exclusively in hematopoietic cells that is involved in cell signaling and regulation of the cytoskeleton. WAS platelets show decreased aggregation to ADP, epinephrine, and collagen and reduced numbers of dense granules.[25]

Bernard-Soulier syndrome (BSS) is characterized by both thrombocytopenia and dysfunctional platelets and is discussed later in this chapter. **May-Hegglin anomaly (MHA)** is characterized by a moderate macrothrombocytopenia and Döhle-like inclusions in leukocytes and is one of a group of disorders associated with abnormalities of the *MYH9* gene (nonmuscle myosin gene). MHA is discussed in Chapter 21.

☑ CHECKPOINT 33-5

What is the pathophysiology of thrombocytopenia in megaloblastic anemia?

Increased Splenic Sequestration

The spleen normally stores approximately one-third of the platelets produced and released by the bone marrow in a pool that is in equilibrium with circulating platelets. In some conditions in which the spleen is enlarged (congestive splenomegaly and reactive splenomegaly or hypersplenism), the proportion of platelets sequestered also is increased and can reach 90% of the total platelet mass.[26] *Hypersplenism* is a condition in which the spleen is enlarged as well as hyperactive. The enlarged spleen can simultaneously sequester erythrocytes and neutrophils. Because the bone marrow production remains constant, the number of circulating platelets decreases. The platelet count is usually above 20×10^9/L, and symptoms of excess bleeding usually are not seen.

Most causes of thrombocytopenia resulting from splenomegaly are complicated by other factors that also contribute to it. It is rare for splenomegaly to occur as an isolated event. Examples of diseases in which splenomegaly is found include hepatic cirrhosis with portal hypertension, hemolytic anemia, infections, Gaucher's disease and other storage diseases, Felty's syndrome, leukemias, and lymphomas. Bone marrow infiltration with malignant cells can complicate the decreased platelet count in lymphomas.

Some myeloproliferative neoplasms (MPNs) are characterized by splenomegaly but increased platelet counts. In these conditions, the number of platelets sequestered increases, but the spleen also may be involved in producing platelets (extramedullary hematopoiesis).

Dilutional Thrombocytopenia

Platelet counts decrease by 30–70% following major surgery because of a combination of hemodilution associated with fluids infused during the procedure and increased consumption.[21] Patients who experience massive hemorrhage requiring replacement of 10 or more units of blood within 24 hours can develop thrombocytopenia if only stored banked blood is used for transfusion. Because of the relatively short half-life of platelets, minimal viable platelets are in banked blood. Certain coagulation factors are also deficient in stored blood, possibly contributing to the bleeding diathesis. Recommendations for patients receiving massive transfusions are to monitor the platelet count, PT, and APTT and to administer platelet transfusions to maintain the platelet count at 75×10^9/L.[27]

Conditions with Multiple Mechanisms of Thrombocytopenia

Thrombocytopenia in some conditions is the result of more than one mechanism (Table 33-9 ★). In alcoholic patients without cirrhosis, the major effect of ethanol is on the platelets. When cirrhosis is present, coagulation factors can also be affected. Alcohol reduces platelet numbers and causes defects of aggregation, secretion, and procoagulant activity. Platelet production is suppressed by a direct toxic effect of alcohol on the bone marrow, which can be compounded by ineffective production associated with a deficiency of folate. Also, decreased TPO production by the liver contributes to a reduced thrombopoiesis. Patients with cirrhosis have enlarged spleens as the result of passive congestion, also contributing to thrombocytopenia.

★ **TABLE 33-9** Conditions with Multiple Mechanisms of Thrombocytopenia

Alcoholism
- Suppressed platelet production
- Ineffective platelet production
- Increased destruction
- Splenomegaly

Lymphoproliferative disease
- Impaired production
- Immune destruction
- Splenomegaly

Cardiopulmonary bypass surgery
- Mechanical destruction
- Increased utilization
- Dilutional thrombocytopenia
- Inadequate neutralization of heparin

In lymphoproliferative disease, when the tumor affects the bone marrow, production of platelets is impaired. Additionally, the production of autoantibodies can enhance platelet destruction. If splenomegaly is present, increased sequestration by the spleen can contribute to the thrombocytopenia.

During cardiopulmonary bypass surgery, the patient's blood circulates through a pump outside the body to be oxygenated, altering hemostasis in a variety of ways. The platelet count decreases to approximately one-half of the presurgical level because of the dilution by IV fluids and donor blood if transfusions are necessary. Platelet counts usually remain above 100×10^9/L during the procedure but can take several days to correct to the patient's normal concentration. Platelets can be activated or mechanically damaged by the artificial surfaces encountered during the procedure.[1] The extent of platelet dysfunction correlates with the duration of the surgical procedure. Additional effects of extracorporeal circulation are discussed in later sections.

CASE STUDY (continued from page 669)

Mohammed's laboratory results were:

Hematology	Hemostasis
WBC 10.5×10^9/L	Prothrombin time 11.2 sec
RBC 2.3×10^{12}/L	APTT 25.6 sec
Hb 6.6 g/dL (66 g/L)	
Hct 0.193 L/L (19.3%)	
Platelet count 133×10^9/L	

3. Explain how these laboratory tests confirm that the patient's bleeding is related to a defect in primary hemostasis rather than secondary hemostasis.
4. Is the bleeding more likely related to problems with the vascular system or with platelets? Why?
5. Is this profuse bleeding with the presence of petechiae consistent with the platelet count? Why?
6. What additional testing would be helpful to identify the cause of the patient's excess bleeding?

Thrombocytosis

Thrombocytosis is the general term for a platelet count that is elevated above the established reference interval. On the peripheral smear, >20 platelets are seen per 100× oil immersion field, and they can appear in large clumps on capillary or first drop smears. They also can be more concentrated on the featheredge of a smear.

Sustained increases in platelet numbers are the result of increased production by the bone marrow because the platelet's life span is not increased. Bone marrow megakaryocytes are increased on histologic sections and can be found in clusters. On buffy coat smears from the marrow, significant numbers of megakaryocytes can be present on the featheredge and throughout the body of the smear. Refer to Table 33-10 ★ for primary and secondary conditions associated with thrombocytosis.

Primary Thrombocytosis

Primary thrombocytosis can be either familial or acquired. In acquired primary thrombocytosis, megakaryocyte proliferation and maturation bypass the normal regulatory mechanisms. Uncontrolled or autonomous production of megakaryocytes in the bone marrow results in a marked increase in the number of circulating platelets. The platelet count is usually $>1000 \times 10^9$/L. In the bone marrow, megakaryocyte hyperplasia as well as giant megakaryocytes with increased ploidy are seen, and hematopoiesis is clonal. In familial thrombocytosis, hematopoiesis is polyclonal. Identified genetic mutations include genetic abnormalities of both thrombopoietin production, and the thrombopoietin receptor.[28]

Acquired primary thrombocytosis occurs in both the chronic myeloproliferative neoplasms (MPNs) and myelodysplasia (Chapters 24 and 25). In essential thrombocythemia (Chapter 24), the megakaryocyte lineage predominates. Thirty-five percent of patients with refractory anemia with ringed sideroblasts (RARS) and some patients with the 5q− syndrome have thrombocytosis, but the platelet count is usually $<1000 \times 10^9$/L (Chapter 25).[28]

Patients with MPNs can have either hemorrhagic or thrombotic episodes; their cause is not always known. Although thrombotic complications are slightly more frequent than bleeding symptoms in general, patients with chronic myelogenous leukemia only rarely develop thrombosis. Hemorrhagic symptoms are present in approximately 25% of patients despite the increased numbers of platelets. Epistaxis and bleeding from the gastrointestinal tract and from other mucous membranes can be quite profuse.

Abnormal platelet aggregation has been demonstrated most frequently with epinephrine but can also be abnormal with ADP and collagen.[28] Screening tests for clotting factors are normal.

★ TABLE 33-10 Causes of Thrombocytosis

Primary thrombocytosis
- Familial thrombocytosis
- Essential thrombocythemia
- Chronic myelogenous leukemia
- Polycythemia vera
- Chronic idiopathic myelofibrosis
- Refractory anemia with ringed sideroblasts
- 5q− syndrome

Secondary (reactive) thrombocytosis
- Acute hemorrhage
- Surgery
- Postsplenectomy
- Recovery from thrombocytopenia
  - Alcohol-induced thrombocytopenia
  - Chemotherapeutic drugs
  - Therapy of cobalamin deficiency
- Malignant diseases
- Chronic inflammatory diseases
- Iron-deficiency anemia
- Hemolytic anemia

Transient thrombocytosis
- Vigorous exercise
- Epinephrine
- Childbirth

Secondary (Reactive) Thrombocytosis and Transient Thrombocytosis

Secondary thrombocytosis is also called **reactive thrombocytosis** because another disease or condition causes the increased platelet count by normal regulatory mechanisms. The platelet count returns to the reference interval by treating the primary condition. The clinical picture is generally related to the underlying condition rather than the thrombocytosis.

Differentiating primary from secondary thrombocytosis can be difficult. Although platelet counts are usually $<1000 \times 10^9$/L in reactive thrombocytosis, they can be as high as in primary thrombocytosis. The bleeding time and platelet aggregation tests are normal, and hemorrhagic and thrombotic complications are infrequent.[28] IL-6 is increased in most patients with secondary thrombocytosis but not in primary thrombocytosis; however, routine testing procedures are generally not available for this cytokine.

Several conditions associated with reactive thrombocytosis are shown in Table 33-10. The etiology of the increased platelet count varies. After acute hemorrhage or surgery, the platelet count can rise to 600×10^9/L or more but returns to the reference interval within a short time. After splenectomy, the platelet count rises, sometimes to $>1000 \times 10^9$/L and can remain elevated for several months. The rise in platelet count exceeds that expected for the loss of the splenic reservoir function, indicating that the spleen may play a role in regulating platelet production. Rebound thrombocytosis can follow recovery from thrombocytopenia in alcoholics and patients on chemotherapeutic drugs or after therapy for vitamin B_{12} deficiency. Platelet counts in patients with iron deficiency anemia vary from thrombocytopenic to $>1000 \times 10^9$/L. In adults with iron deficiency anemia resulting from chronic blood loss, thrombocytosis is commonly reported (50–75% of patients), and the platelet count returns to normal after iron replacement. Thrombocytosis is thought to occur in hemolytic anemias because of the stimulation of the bone marrow by hematopoietic growth factors to produce erythrocytes resulting in stimulation of the megakaryocytic lineage as well.

In transient thrombocytosis, platelet counts rise transiently in the conditions found in Table 33-10.

☑ CHECKPOINT 33-6

Explain why primary thrombocytosis is often associated with abnormal platelet function, whereas secondary thrombocytosis is not.

Artifacts in the Quantitative Measurement of Platelets

The laboratory must be aware of artifacts responsible for erroneously (spuriously) low or high platelet counts in automated electronic platelet counting.[14] Recognition of these artifacts can prevent misdiagnosis and inappropriate or unnecessary diagnostic procedures and therapy. In many cases, examining a blood smear can reduce these errors. Pseudothrombocytopenia is an in vitro artifact of automated cell counting seen when blood is collected in ethylenediaminetetraacetic acid (EDTA). Aggregates of platelets or platelet satellitism (platelets bound to neutrophils) are common findings on the peripheral smear. See Chapters 9, 10, and 43 for a description of these artifacts in more detail as well as suggestions for resolving the discrepancies.

CASE STUDY (continued from page 678)

Mohammed's hematology report included a finding that at least 65% of the platelets were large and giant forms with intense granulation.

7. Is there a possibility that the patient's platelet count is spuriously increased or decreased? Why?

Qualitative (Functional) Platelet Disorders

The functions of platelets in primary hemostasis were discussed in Chapter 31. The platelet's roles in hemostasis include platelet adhesion, contraction, release of the contents of the α-granules (αGs), dense granules and lysosomes, generation of thromboxane A_2, aggregation, and procoagulant activity. Inherited or acquired abnormalities in any phase of platelet function can lead to defective formation of a primary hemostatic plug and abnormal bleeding.

Clinical symptoms vary from asymptomatic to mild, easy bruisability to severe, life-threatening hemorrhaging depending on the nature of the defect. The type of bleeding is similar to that seen in thrombocytopenic disorders. Common manifestations include petechiae, easy and spontaneous bruising, bleeding from mucous membranes such as the nose or gastrointestinal tract, abnormal vaginal bleeding, and prolonged bleeding from trauma.

Laboratory screening test results are similar to those found in thrombocytopenia except that the platelet count is usually normal. A mildly decreased platelet count, however, is characteristic of some conditions. Screening tests for coagulation factors (PT, APTT) and tests for fibrinolysis are normal. Special laboratory tests for platelet function reflect the nature of the platelet defect.

Platelet functional disorders are classified as hereditary or acquired. The inheritance pattern in the hereditary types is usually autosomal recessive.

Hereditary Disorders of Platelet Function

Hereditary platelet defects are rarely encountered clinically. However, much of the present knowledge of platelet function has been derived from the study of patients with such anomalies. Defects in each phase of platelet function have been described (Table 33-11 ★). A convenient classification scheme is based on the steps in platelet function. Abnormalities in function can be related to affected portions of the platelet ultrastructure (Figure 33-6 ■).

Disorders of Platelet Adhesion

Platelet adhesion to exposed subendothelium initiates the events of primary hemostasis. There are a number of adhesive proteins in the subendothelium to which platelets have receptors, including fibronectin, laminin, vitronectin, and collagen (Chapter 31). Of these, adhesion to collagen appears to be essential for firm platelet adhesion in vivo.[29] Adhesion to collagen at the high shear rates encountered in arterioles

★ TABLE 33-11 Inherited Disorders of Platelet Function

Disorders of adhesion (defects in platelet-vessel wall interaction)
- von Willebrand disease (deficiency/defect in plasma VWF)
- Bernard-Soulier syndrome (deficiency/defect in GPIb/IX)
- Deficiency/defect of collagen receptors (GPIa/IIa, GPVI)

Disorders of aggregation (defects in platelet–platelet interaction)
- Congenital afibrinogenemia (deficiency of plasma fibrinogen)
- Glanzmann thrombasthenia (deficiency/defect in GPIIb/IIIa)

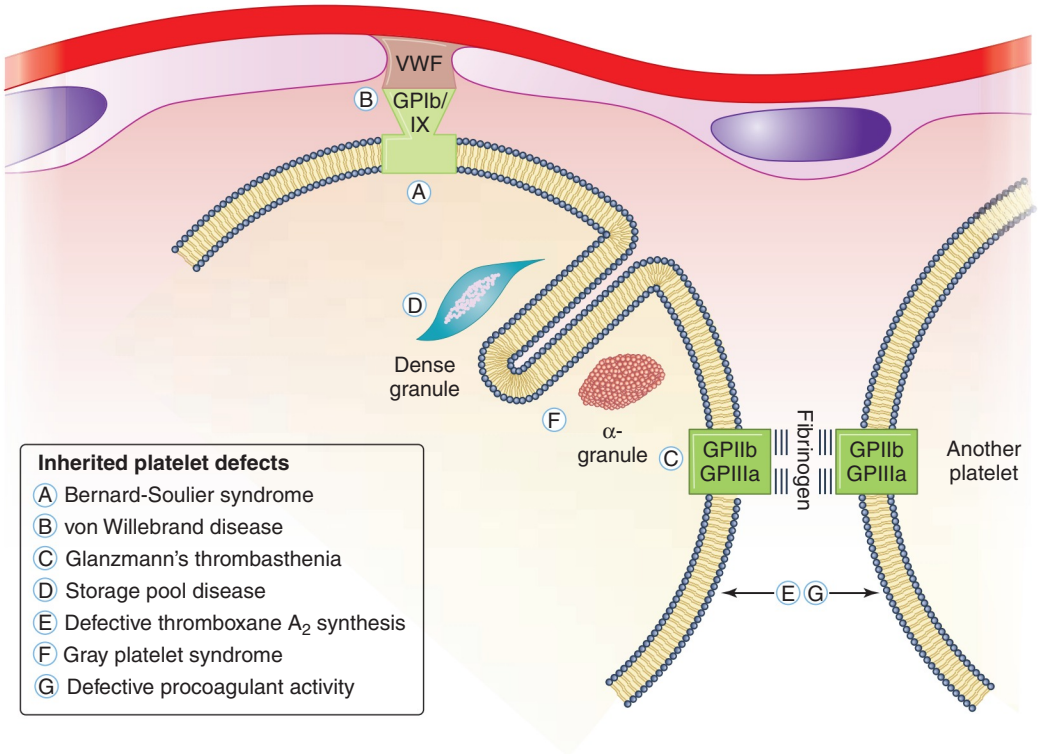
Disorders of platelet secretion and abnormalities of granules
- Storage pool deficiency (δSPD; αSPD)
- Quebec platelet disorder

Disorders of platelet secretion and signal transduction
- Receptor defects (defects in platelet–agonist interaction) (Receptor defects: TXA_2, ADP, collagen, epinephrine, serotonin)
- Defects in G-protein activation
- Defects in phosphatidylinositol metabolism (phospholipase C deficiency)
- Defects in protein phosphorylation (PKC deficiency)
- Abnormalities in arachidonic acid pathways and TXA_2 synthesis (deficiency of phospholipase A2, cyclooxygenase, thromboxane synthase)

Disorders of platelet coagulant–protein interaction
- Scott syndrome

and the microcirculation requires the presence of both an adequate amount of functional von Willebrand factor (VWF) and the presence of functional GPIb/IX on the platelet membrane (Chapter 31). VWF acts as a bridge binding to the platelet via GPIb/IX and to collagen. Deficiencies of either VWF or GPIb/IX result in defective in vivo platelet adhesion. Collagen is also a platelet agonist and plays an important role in initiating platelet function; thus, aggregation and formation of the primary hemostatic plug are also defective.

Bernard-Soulier Syndrome. A rare autosomal-recessive disorder, BSS was first described by Bernard and Soulier in 1948.[30] The incidence of BSS is <1 in 1,000,000.[31] It is characterized by a



■ **FIGURE 33-6** Ultrastructural components associated with inherited disorders of platelet function.

★ **TABLE 33-12** Laboratory Test Results in Selected Platelet Disorders

Disorder	Defective Platelet Component	Platelet Count	Bleeding Time Test	Platelet Aggregation	Closure Time (PFA)	Other
Bernard-Soulier syndrome	Glycoprotein Ib/IX	Normal or decreased	Increased	Abnormal with ristocetin Normal with ADP, collagen, epinephrine	Increased	Giant platelets
Glanzmann thrombasthenia	Glycoprotein IIb/IIIa	Normal	Increased	Abnormal with ADP, collagen, epinephrine Normal with ristocetin	Increased	
δ-storage pool disease	Dense granule deficiency	Normal	Increased	Abnormal secondary aggregation with ADP, epinephrine; abnormal with collagen Normal primary aggregation	Variable	
Gray platelet syndrome	α-granule deficiency	Variably decreased	Variable	Normal	Variable	Agranular platelet
Defective thromboxane A_2 synthesis	Deficiency of cyclooxygenase or TXA_2 synthase	Normal	Increased	Abnormal secondary aggregation with ADP, abnormal with collagen Normal primary aggregation	Increased	

PFA = platelet function analyzer

moderate to severe thrombocytopenia, giant platelets, and abnormal platelet function. Homozygous patients have a lifelong bleeding tendency that can begin in infancy. Bleeding symptoms are similar to those described for thrombocytopenia. Heterozygous persons usually have no significant bleeding symptoms.[31]

The defect in BSS is a quantitative decrease or abnormal function of the GPIb/IX/V complex. It results from a mutation in GPIbα, GPIbβ, or GPIX. Although GPV is missing from the membrane of BSS platelets, this is apparently because of the loss of the GPIb/IX complex. Mutations in the gene for GPV do not result in a BSS phenotype. Lack of functional GPIb/IX prevents interaction of the platelets with VWF and subsequent platelet adhesion to collagen (Figure 33-6a). More than 50 genetic mutations associated with BSS have been identified.[29]

Laboratory tests are required to diagnose this disease and differentiate it from other platelet functional disorders and other causes of macrothrombocytopenia (Table 33-12 ★). Thrombocytopenia can be variable, with some patients having nearly normal counts and occasional patients having platelet counts of $<20 \times 10^9$/L.[29] Thirty to eighty percent of platelets have mean diameters >3.5 mcM (μm) and occasionally up to 20 mcM (Figure 33-7 ■). The significance and cause of the macrothrombocytopenia is not known. Bleeding is prolonged more than expected for the degree of thrombocytopenia, indicating a coexistent disorder of platelet function. Platelet aggregation studies are normal with ADP, collagen, and epinephrine. However, agglutination with ristocetin, which requires VWF and GPIb/IX, is abnormal[29, 31] (Figure 33-8a ■) (Chapter 36).

Similar platelet aggregation results are obtained in patients with classic VWD. To differentiate them, a modification of the ristocetin agglutination test is used. The addition of VWF (e.g., normal plasma) to the patient's platelet suspension does not correct the abnormal ristocetin agglutination in BSS patients but does correct the agglutination defect in VWD (Figure 33-8b). Also, flow cytometry can characterize the platelet surface GPIb/IX proteins (CD42b/CD42a).

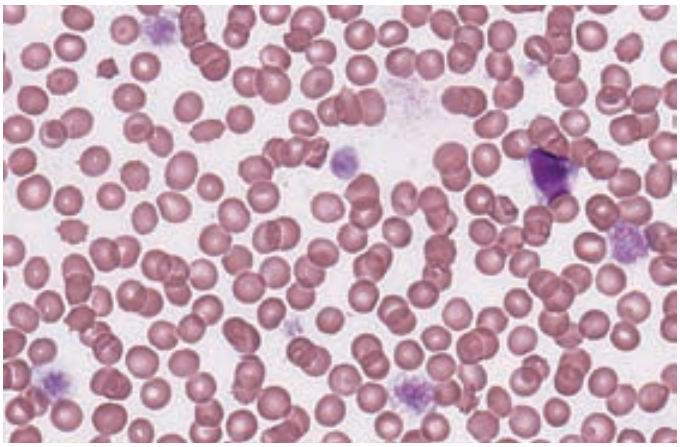
BSS has no specific treatment. Supportive measures such as erythrocyte and platelet transfusions are used as needed. Bone marrow transplantation from human leukocyte antigen (HLA)–identical donors have successfully corrected the hemostatic defect in BSS patients.

von Willebrand Disease. VWD is characterized by a decrease in production of VWF or of a dysfunctional protein (Figure 33-6b). Because it is a plasma protein disorder rather than a platelet functional disorder, VWD is discussed in Chapter 34.

Defects of Collagen Receptors. In addition to platelet adhesion to collagen mediated by VWF/GPIb/IX, platelets contain several other membrane receptors capable of direct collagen binding. Both GPIa/IIa and GPVI participate in platelet interactions with collagen, and bleeding defects have been reported in individuals with decreased levels of either receptor.[32]

Disorders of Platelet Aggregation

Platelet aggregation requires the presence of fibrinogen and the GPIIb/IIIa receptor on the platelet membrane. In the absence of either of these components, platelets do not interact with one another



■ **FIGURE 33-7** Platelet morphology in Bernard-Soulier syndrome (peripheral blood, Wright-Giemsa stain, 1000× magnification).

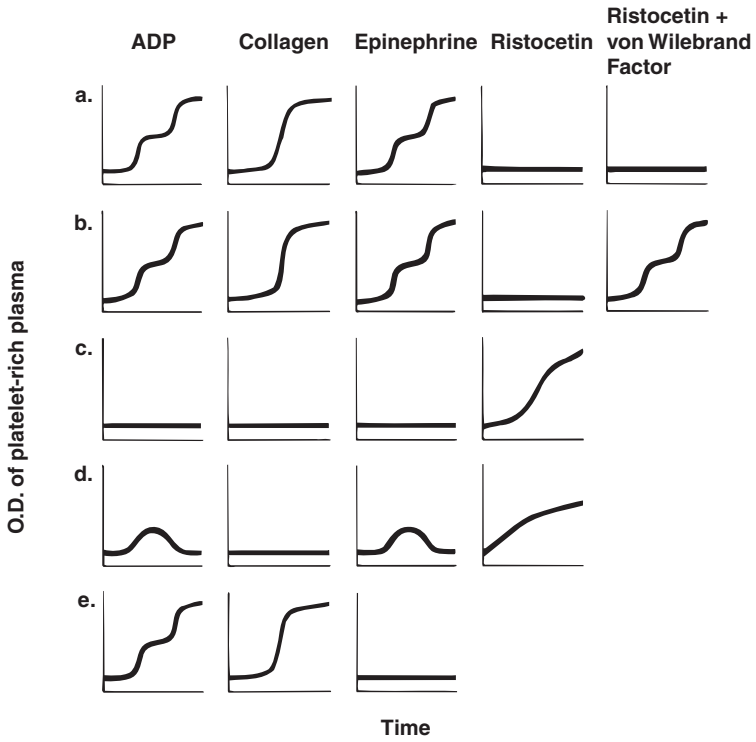


FIGURE 33-8 Platelet aggregation patterns in disorders of platelet function. **(a)** Bernard-Soulier syndrome. **(b)** von Willebrand disease. **(c)** Glanzmann thrombasthenia. **(d)** δ-storage pool disease, aspirin ingestion, uremia, thromboxane A_2 deficiency. **(e)** Myeloproliferative neoplasm.

to produce primary or secondary aggregation. The congenital disorder in which the GPIIb/IIIa complex is defective is **Glanzmann thrombasthenia (GT)**. The absence of fibrinogen is discussed in Chapter 34.

Glanzmann Thrombasthenia. GT is a rare autosomal recessive disease first described in 1918.[33] Clinical manifestations are apparent only in homozygotes.[34] Globally, GT is extremely rare, but it can be clustered in populations where consanguineous marriages are common.[29] Platelets of patients with GT are deficient in the GPIIb/IIIa complex (Figure 33-6c), the site of attachment of fibrinogen to the platelet surface. Fibrinogen "bridges" two platelets in the presence of calcium, initiating platelet aggregation (Chapter 31). Some patients have a deficiency of fibrinogen in the αGs, presumably because of decreased GPIIb/IIIa-mediated endocytosis of fibrinogen from the plasma.

Glycoproteins IIb and IIIa are encoded by separate genes closely linked on the long arm of chromosome 17. A deficiency of the whole complex in the platelet membrane occurs when either gene is defective. Approximately 200 different molecular defects of the two genes have been identified.[29] Three subtypes of GT have been described: GT Type I is characterized by undetectable or trace amounts (<5%) of GPIIb/IIIa and the absence of αG fibrinogen; GT type II patients have GPIIb/IIIa of 10–20% of normal, and αG fibrinogen is present; GT type III is a qualitative defect of GPIIb/IIIa with laboratory diagnostic features of GT except that GPIIb/IIIa levels are 50–100% of normal. Obligate carriers (heterozygotes) of a quantitative defect have 50–60% of the complex but are phenotypically normal.[29]

Platelet aggregation does not occur in GT, but aspects of platelet function that do not depend on aggregation, such as adhesion and secretion, are usually normal. Additional abnormalities of platelets occur in patients with GT, including deficiencies of antigens such as HPA-1a and HPA-1b that are normally present on the GPIIb/IIIa complex.

Bleeding symptoms can begin in infancy and involve superficial areas of the body characteristic of platelet abnormalities. Although they are usually described as moderate in severity, deaths have occurred. Complications from GT can arise during childbirth, tooth extraction, or any surgical procedure.[35]

Laboratory tests are necessary to differentiate this disorder from other platelet defects (Table 33-12). The platelet count and morphology are normal, but the bleeding time is markedly prolonged, and clot retraction is abnormal, indicating platelet dysfunction. Platelet aggregation tests show no response to agonists such as ADP, epinephrine, or collagen because the platelets lack the site of attachment of fibrinogen; thus, aggregation cannot occur. However, normal agglutination with ristocetin occurs (Figure 33-8c). Deficiencies of GPIIb or IIIa are identified by flow cytometry of platelets utilizing anti-CD41 or anti-CD61 antibodies or observing a quantitative decrease of the proteins using polyacrylamide gel electrophoresis to separate platelet glycoproteins.

No specific treatment is available for thrombasthenic patients. Supportive platelet transfusions are used when needed, and bone marrow transplantation has been performed in rare cases.[36] In Europe, **recombinant FVIIa (rFVIIa)** is approved for treatment of GT patients with antibodies to GPIIb/IIIa and/or human leukocyte antigen(s) (HLA) with past or present refractoriness to platelet transfusions.[37] As of January 2012, this is an off-label use in the United States.

Disorders of Platelet Secretion, Granule Abnormalities, and Signal Transduction

Disorders of platelet secretion and signal transduction include a heterogeneous group of disorders that have in common the impaired secretion of granule contents and abnormal aggregation during platelet activation. In aggregation studies, the second wave of aggregation has been found to be reduced or absent in some of these disorders. The abnormal platelet functions occur either because the granules or their contents are decreased (storage pool deficiency) or because of an abnormality in the activation mechanisms regulating secretion and aggregation.[29] Symptoms vary from moderate to mild bleeding tendencies depending on the abnormality. Manifestations can be easy bruising, hemorrhage, and excess bleeding after surgery or childbirth. The platelet count is normal.

Deficiencies of Dense Granules (**δ-Storage Pool Disease/δSPD**). δSPD include a heterogeneous group of disorders of variable inheritance that share the common feature of DG deficiency. The platelets in these disorders show a decrease or absence of platelet DG by electron microscopy (Figure 33-6d), but the morphologic appearance of the platelets on stained peripheral blood smears is normal. The bleeding time is usually prolonged (Table 33-12), and platelet aggregation tests are abnormal with ADP, epinephrine (normal primary wave of aggregation, absent or blunted secondary wave) and low levels of collagen (markedly reduced response) (Figure 33-8d). The platelet aggregation abnormalities result from the lack of ADP release from the DG so that secondary aggregation does not occur with ADP or epinephrine. The platelet aggregation curve induced by collagen is normally produced by release of endogenous DG products ("secondary aggregation" only) and is therefore absent. The ratio of total platelet ATP:ADP is increased (>2.5).[29] Platelet agglutination studies using ristocetin demonstrate normal results.

Deficiencies of DG occur as one of the features of several rare autosomal recessive disorders including Chediak-Higashi syndrome, Hermansky-Pudlak syndrome, Wiskott-Aldrich syndrome, and TAR syndrome.

Gray Platelet Syndrome (GPS). Selective deficiency of platelet αGs is called the **gray platelet syndrome** or **α-storage pool disease**. Because αGs are so numerous, their absence causes the platelets to appear agranular on a peripheral blood smear. By electron microscopy, αGs are absent or markedly decreased. Studies indicate that megakaryocyte synthesis of the proteins found in αGs is normal, and the defect may be in targeting endogenously synthesized proteins to the developing αGs.[29] In contrast to δ-storage pool disease, platelet aggregation studies are generally normal, and clinical manifestations are usually mild. Patients generally have a mild thrombocytopenia and macrothrombocytes (Table 33-12). Mutations in the *NBEAL2* gene recently have been identified as the genetic cause of recessively inherited GPS.[38] NBEAL2 is believed to be involved in vesicular trafficking and αG development.

Quebec Platelet Disorder. **Quebec platelet disorder** is a rare autosomal dominant disorder associated with abnormal proteolysis of αG proteins resulting from increased levels of platelet urinary-type plasminogen activator in platelet αGs.[29] In contrast to GPS, the structure of the αG is preserved, and platelets are morphologically normal by light microscopy. Platelet counts often are moderately decreased, and patients experience a variable bleeding history.

Abnormalities of the Platelet Secretory Mechanism. These disorders are typically associated with a mild to moderate bleeding history. Patients usually have absence of the second wave of platelet aggregation with ADP or epinephrine and decreased aggregation with collagen. These disorders must be differentiated from acquired abnormalities of platelet secretion induced by drugs such as aspirin (see the section "Acquired Disorders of Platelet Function/Aspirin"). Although individually rare, these disorders as a group are far more common than GT, BSS, or SPD.

Defective Thromboxane A_2 (TXA_2) Synthesis. Liberation of arachidonic acid from membrane phospholipids and its conversion to TXA_2 is an important positive feedback loop enhancing the platelet activation process (Chapter 31). A defect in the pathway of TXA_2 synthesis produces a platelet aggregation pattern similar to that seen in δ-storage pool disease (Figure 33-6d, Table 33-12). Defects can occur in the liberation of arachidonic acid from phospholipids (phospholipase A_2 deficiency) and in TXA_2 synthesis (cyclooxygenase deficiency or thromboxane synthase deficiency).[29] Platelet secretion and secondary aggregation do not occur.

Signal Transduction Defects. Various heterogeneous disorders are grouped together as signal transduction defects. The defect can involve platelet–agonist interaction (i.e., defect of the platelet receptor for a specific agonist). In these disorders, patients typically have impaired platelet responses to a single agonist in platelet aggregation assays. Documented receptor defects include receptors for TXA_2 (TPα), ADP ($P2Y_{12}$), collagen (GPIa/IIa or GPVI), epinephrine (α_2-adrenergic receptors [αARs]), and serotonin.[29,39] See Table 33-11 for other potential defects in platelet secretion and signal transduction.

Disorders of Platelet Procoagulant Activity

Defective procoagulant activity of platelets has been described as an additional finding in several of the previously mentioned disorders and as a single entity in some patients. **Scott syndrome** is a rare autosomal recessive disorder characterized by abnormal Ca^{++}-induced phospholipid scrambling. The activated platelets secrete and aggregate normally but fail to transport phosphatidyl serine from the inner to the outer phospholipid leaflet of the membrane. This phospholipid redistribution is required for activated platelets to be able to assemble the tenase and prothrombinase complexes, and thrombin formation is defective.[29,40]

The membrane activity thought to be responsible for this phospholipid movement has been named *scramblase* (Chapter 31). Mutations in the gene *TMEM16F* recently were associated with Scott syndrome, suggesting that this protein is part of the scramblast complex.[41]

☑ CHECKPOINT 33-7

A patient is having laboratory tests to evaluate for a bleeding phenotype suggestive of a defect of primary hemostasis. Platelet aggregation test results indicate normal aggregation with thrombin, epinephrine, and ADP but defective agglutination with ristocetin. What aspect of platelet function is defective? Name two clinical conditions that would produce these test results.

Acquired Disorders of Platelet Function

Platelet dysfunction is induced in a variety of conditions and with the ingestion of certain drugs. Clinical manifestations and the results of laboratory tests vary with the cause and the resulting effect on the platelet mechanism.

Chronic Renal Failure

A bleeding tendency in uremia recognized for many years was first associated with a platelet functional abnormality in 1956. The platelet defect's pathogenesis and severity are related to the accumulation of waste products in the blood, although which of the metabolites produce harmful effects is unclear. The bleeding time is prolonged and seems to correlate with the severity of the renal failure. The platelet aggregation test with collagen and secondary aggregation with ADP and epinephrine is decreased, indicating an abnormal secretory response (Figure 33-8d). Platelet procoagulant activity also is defective.

Bleeding symptoms in uremic patients can be severe. Ecchymoses, gastrointestinal bleeding, and hemorrhages into serous cavities can be seen. Clinical symptoms decrease with dialysis treatment. Cryoprecipitate transfusion has also been proposed as treatment in this setting.

Hematologic Disorders

The bleeding and thrombotic problems in myeloproliferative neoplasms (MPNs) were described earlier in this chapter. An abnormal response to epinephrine, ADP, or collagen in the platelet aggregation test is often seen. Other platelet abnormalities that have been reported in some patients include decreased synthesis and/or response to TXA_2, reduced αGs or DGs, decreased membrane glycoproteins, and decreased procoagulant activity[42] (Figure 33-8e).

Defective platelet aggregation has been noted in patients with acute leukemia and myelodysplasia. Bleeding problems in these conditions, however, usually result from thrombocytopenia. Variable defects of platelet function have also been reported.[42]

In addition to impaired vascular function in patients with dysproteinemias such as multiple myeloma and macroglobulinemia described earlier, abnormal platelet function is observed (Chapter 28). Severe bleeding symptoms can result. Thrombocytopenia and hyperviscosity are the major causes of the bleeding tendency, but platelet function is abnormal because the paraprotein coats the platelet surface and interferes with the membrane reactions of platelet activation. The bleeding symptoms and the abnormal platelet function are proportional to the amount of abnormal protein in the plasma. Abnormal tests include platelet aggregation and platelet procoagulant activity. The results of the abnormal tests, however, do not always correlate with the severity of clinical bleeding.

Drugs

Many drugs have been shown to contribute to platelet dysfunction. The effect of drugs on platelets of persons with normal hemostatic function is usually clinically unnoticeable. However, those with abnormalities of the hemostatic system are at increased risk for developing severe bleeding symptoms.

Drugs can variably alter the platelet aggregation studies, but the effects on these laboratory tests do not necessarily correlate with clinical symptoms. The mechanisms of inhibiting platelet function are variable and not always completely understood.

Aspirin. Aspirin affects platelet function by irreversibly acetylating, and thus inactivating, the platelet COX-1 enzyme, thereby preventing the formation and release of TXA_2. As a result, platelet secretion is decreased. A single 80-mg "baby aspirin" taken daily can completely block platelet TXA_2 production.[42] The platelets affected by aspirin continue to circulate but are nonfunctional. Laboratory tests of platelet function, therefore, can be altered in patients taking aspirin. Platelet function tests return to normal as new platelets are produced and released from the marrow. Tests typically become normal 7 days after the last dose.

In persons with a functional abnormality of platelets (either hereditary or acquired) or VWD, aspirin ingestion can lead to serious bleeding complications. It is recommended that patients should not ingest aspirin or any of the numerous aspirin-containing products for 7 days before having platelet function tests or most surgical procedures.

Abnormalities in platelet aggregation tests reflect the lack of TXA_2 synthesis and are similar to results from patients with hereditary deficiencies of enzymes in this pathway. A first wave of aggregation is seen with ADP and epinephrine, but no secondary wave is present. Aggregation does not occur with collagen, and agglutination is normal with ristocetin (Figure 33-8d). For further discussion of the effects of drugs on platelet function testing, see Chapter 36.

☑ CHECKPOINT 33-8

Why are the bleeding time test and closure time abnormal for up to 7 days following ingestion of aspirin?

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs). NSAIDs that inhibit COX-1 can also inhibit platelet function. Examples include ibuprofen, naproxen, indomethacin, and sulfinpyrazone (among others). However, COX-1 inhibition by these drugs is reversible, and usually short lived (<72 hours). Selective COX-2 inhibitors, such as celecoxib, have no antiplatelet activity. Acetaminophen does not impair platelet function.

Alcohol. Ingestion of large amounts of alcohol over a long period of time can lead to platelet dysfunction in some individuals. The platelet aggregation test can show decreased primary aggregation with ADP.

Antibiotics. Antibiotics, particularly penicillins and cephalosporins (which affect bacterial cell wall synthesis), alter platelet function. Patients taking these drugs show no aggregation with ADP either in the primary or secondary wave. The drug is believed to coat the platelet membrane, blocking ADP and epinephrine receptors and resulting in platelet inability to respond to agonist. Serious bleeding complications can occur when the drugs are given in high doses, and for long periods of time.

Cardiopulmonary Bypass Surgery

In addition to thrombocytopenia as discussed previously, platelet function is altered during cardiopulmonary bypass surgery. The platelets are believed to become activated by the abnormal surfaces in the extracorporal pump to which they are exposed.

Significant bleeding develops in approximately 3–5% of patients after bypass surgery. In approximately 50% of these cases, the bleeding occurs because of inadequate surgical technique and can require additional surgery to correct. The remaining 50% can result from a variety of defects of hemostasis, the most common of which is acquired abnormal platelet function.

Flow cytometry has been used to study platelet activation during cardiopulmonary bypass surgery in pediatric patients. Monoclonal antibodies to CD62P and GPIb/IX are used to correlate levels of these two proteins with the extent of platelet activation and with the risk of bleeding. CD62P is contained within the αG and is expressed on the platelet surface only after platelets have undergone the release reaction. After platelets have been activated, GPIb decreases in density on the platelet surface, whereas CD62P increases.[43]

CASE STUDY *(continued from page 679)*

When asked if he had had bleeding problems in the past, Mohammed recounted having petechiae, lots of bruises off and on since childhood, frequent nosebleeds, and bleeding for a long time after cuts. A platelet function analyzer closure time and platelet aggregation studies were ordered. The closure time was prolonged. Platelet aggregation studies showed normal aggregation with ADP, collagen, epinephrine, and thrombin. Agglutination with ristocetin was abnormal and was not corrected when VWF was added.

8. Is Mohammed's problem more likely acquired or inherited? Why?
9. What is the significance of these platelet function tests?
10. What is Mohammed's most likely condition?
11. What additional testing would be considered?

Summary

Patients affected by disorders of primary hemostasis usually have an imbalance in the hemostatic system and experience bleeding of some type. Although bleeding can occur in any organ, most patients experience excess bleeding from superficial cuts, mucous membranes, easy skin bruising, and the presence of petechiae. Occasional disorders result in excess clotting, that is, thrombosis rather than excess bleeding.

Vascular disorders are diverse in origin and can be inherited or acquired. Vascular disorders are usually diagnosed by excluding other causes of bleeding and observing symptoms consistent with the underlying disorders. Results of routine laboratory tests of hemostasis are usually normal.

Platelet disorders are broadly categorized as quantitative disorders in which the platelet count is too low or too high or as functional abnormalities in which an aspect of platelet function is altered. Thrombocytopenia is caused by conditions that affect the bone marrow production of megakaryocytes, cause increased destruction of platelets after they are released into the peripheral blood, cause splenomegaly, or occur by dilution during transfusion of multiple units of banked blood within a short period of time. Thrombocytosis is seen in MPNs and a number of other diseases. Functional platelet abnormalities are caused by mutations in genes that produce platelet membrane or granule constituents or can be acquired by ingestion of certain drugs such as aspirin. Laboratory tests that are helpful in establishing the cause of a platelet disorder include platelet counts, closure time (PFA-100®), and platelet aggregation studies.

Review Questions

Level I

1. Which of the following platelet counts indicates thrombocytopenia? (Objective 1)
   A. 200×10^9/L
   B. 2000×10^9/L
   C. 200×10^{12}/L
   D. 20.0×10^9/L
2. What laboratory test(s) is (are) most often ordered to screen for abnormalities of the hemostatic system? (Objective 3)
   A. platelet count
   B. prothrombin time
   C. activated partial thromboplastin time
   D. all of the above

3. Which of the following thrombocytopenic conditions is not the result of an immune-mediated mechanism? (Objectives 6, 7)
   A. NAIT
   B. drug-induced thrombocytopenia
   C. post-transfusion purpura
   D. TTP
4. What is the name of purple lesions that are larger than 1 cm in diameter but are *not* raised? (Objective 2)
   A. petechiae
   B. ecchymoses
   C. hematoma
   D. purpura
5. In which hematologic disorder would you expect to observe a decreased platelet count? (Objective 6)
   A. acute leukemia
   B. chronic myelocytic leukemia
   C. hemolytic anemia
   D. iron-deficiency anemia
6. How long should a patient be off aspirin or aspirin-containing products before having platelet function testing? (Objective 9)
   A. 2 hours
   B. 2 days
   C. 7 days
   D. 30 days
7. Bernard Soulier syndrome is caused by: (Objective 10)
   A. mutation in the gene for either GPIIb or GPIIIa
   B. mutation in the von Willebrand factor gene
   C. mutation in the gene for fibrinogen α-, β-, or γ-chain
   D. mutation in the gene for GPIbα, GPIbβ, or GPIX
8. What level of platelet count is associated with a risk of life-threatening bleeding into the central nervous system? (Objective 5)
   A. 200×10^9/L
   B. 100×10^9/L
   C. 50×10^9/L
   D. 5×10^9/L
9. Thrombocytosis is: (Objective 8)
   A. a platelet count within the reference interval for age
   B. an abnormal platelet function
   C. the presence of a blood clot in a leg vein
   D. a platelet count higher than the reference interval
10. ITP (immune thrombocytopenia) is characterized by all of the following *except*: (Objective 7)
    A. in adults, frequently presents following a viral infection
    B. in children, frequently presents as an abrupt onset, acute disease
    C. in adults, can persist for months or years
    D. in children, results in spontaneous remissions in >90% of cases

Level II

Use the following history for questions 1–5.

The mother of a 4-year-old boy noticed several bruises on his arms, legs, and torso. Upon closer examination, she saw several pinpoint, brownish purple spots on his ankles. Just before leaving home for the doctor's office, the child had a moderately severe nosebleed. There had been no previous episodes of this type.

The child's physical examination was unremarkable except for the bruises. The child had not recently taken any medication but was recovering from the chicken pox.

Laboratory results showed:

RBC	4.25×10^{12}/L
Hb	10.8 g/dL (108 g/L)
Hct	34% (.34 L/L)
MCV	80 fL
MCH	25.4 pg
MCHC	30% (.30 L/L)
WBC	5.2×10^9/L
Platelet count	4.0×10^9/L

A bone marrow examination was scheduled but was later cancelled.

1. What disorder is most probably indicated by the boy's history? (Objectives 4, 5)
   A. acute leukemia
   B. Bernard-Soulier syndrome
   C. immune thrombocytopenia
   D. Marfan syndrome
2. If a bone marrow examination had been performed, what morphology would it likely have shown? (Objective 4)
   A. normal to increased numbers of megakaryocytes
   B. a high number of blast cells
   C. decreased numbers of erythrocyte precursors
   D. absence of all myeloid precursors and replacement by fat

3. What is the mechanism of platelet destruction in immune thrombocytopenia? (Objective 4)
   A. lysis by complement in the peripheral blood
   B. increased sequestration of platelets by the spleen
   C. removal of antibody-coated platelets by splenic macrophages
   D. activation and increased utilization by forming aggregates in the blood stream
4. The small purple spots seen on this boy's ankles are most probably: (Objective 2)
   A. petechiae
   B. ecchymoses
   C. allergic purpura
   D. hematoma
5. What is the boy's expected prognosis? (Objective 2)
   A. imminent death from overwhelming infection
   B. complete spontaneous recovery within 6 months
   C. similar problems for the rest of his life
   D. recovery after a long period of steroid therapy
6. Which of the following is characteristic in a patient with Bernard-Soulier syndrome? (Objective 7)
   A. abnormal glycoprotein IIb/IIIa
   B. increased prothrombin time
   C. increased platelet count
   D. abnormal platelet agglutination with ristocetin
7. Which of the following is found in Glanzmann thrombasthenia? (Objective 7)
   A. mutation in the gene for VWF
   B. acquired abnormality of fibrinogen
   C. genetic abnormality of glycoprotein IIb or IIIa
   D. acquired vascular disorder
8. Hereditary telangiectasias is characterized by which of the following? (Objective 3)
   A. abnormal platelet adhesion to collagen
   B. thrombocytosis
   C. deficiency of platelet dense granules
   D. skin lesions that are arterioles connected directly to venules
9. Platelet aggregation studies were performed and showed a primary wave of aggregation with ADP that returned to the baseline with no secondary wave. Which of the following conditions is consistent with these results? (Objectives 7, 8)
   A. senile purpura
   B. chronic immune thrombocytopenia
   C. aspirin effect
   D. Bernard-Soulier syndrome
10. Reactive thrombocytosis is associated with: (Objectives 4, 6)
    A. postsurgery
    B. MPN
    C. aspirin ingestion
    D. heparin therapy

Companion Resources

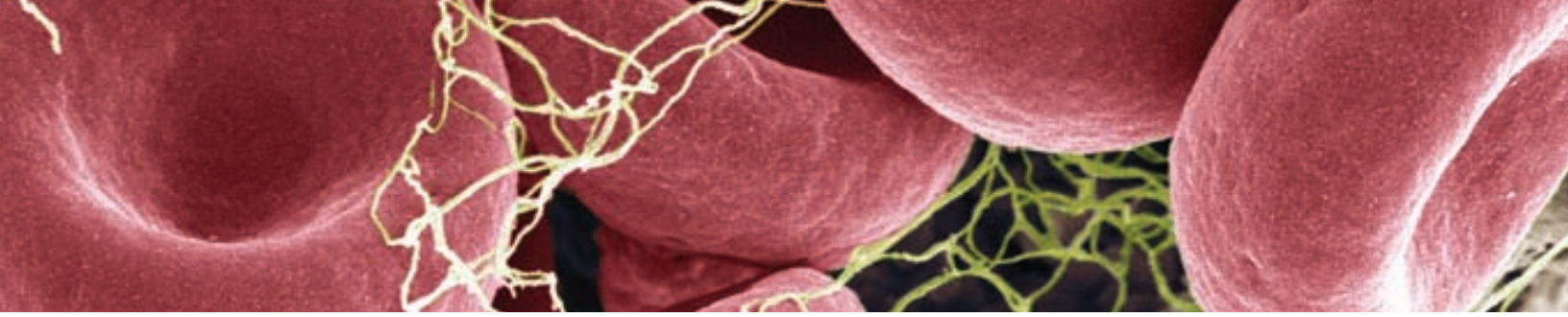
www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Diz-Küçükkaya R, López JA. Acquired disorders of platelet function. In Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone/Elsevier; 2013:1867–82.
2. Aster RH, George JN. Drug-induced thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:776–84.
3. Rao AK, Bennett JS. Acquired disorders of platelet function. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:820–28.
4. Malfait F, DePaepe A. Disorders of connective tissue and extracellular matrix. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:842–54.
5. Steinmann B, Royce P, Superti-Furga A. The Ehlers-Danlos syndrome. In: Royce P, Steinmann B, eds. *Connective Tissue and Its Heritable Disorders*, 2nd ed. New York: Wiley-Liss; 2002:431–523.
6. Callewaert B, Malfait F, Loeys B et al. Ehlers-Danlos syndromes and Marfan syndrome. *Best Pract Res Clin Rheumatol.* 2008;22:165–89.
7. Letarte M, Shovlin CL. Hereditary hemorrhagic telangiectasia. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:855–64.
8. Shovlin CL. Hereditary haemorrhagic telangiectasia: pathogenesis, diagnosis and treatment. *Blood Rev.* 2010;24:203–19.

9. Gautieri A, Uzel S, Vesentini S et al. Molecular and mesoscale mechanisms of Osteogenesis Imperfecta disease in collagen fibrils. *Biophys J*. 2009;97:857–65.
10. Li Q, Jiong Q, Pfendner E et al. Pseudoxanthoma elasticum: clinical phenotyes, molecular genetics and putative pathomechanism. *Exp Dermatol*. 2009;18:1–11.
11. Alexandrescu DT, Gallo RL. The vascular purpuras. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1993–2008.
12. Stone JR, Stone JH. Vasculitides. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1205–15.
13. Lightfoot RW. Palpable purpura: identifying the cause. *Hosp Pract (Off Ed)*. 1992;27:39–47.
14. Bennett JS. Overview of acquired and inherited clinical platelet disorders. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:751–56.
15. Liebman HA, Cines DB, Bussel JB. Immune thrombocytopenia and posttransfusion purpura. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:763–75.
16. Rodeghiero F, Stasi R, Gernsheimer T et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113:2386–393.
17. Arnold DM, Patriquin C, Toltl LJ et al. Diseases of platelet number: immune thrombocytopenia, neonatal alloimmune thrombocytopenia, and posttransfusion purpura. In Hoffman R, Benz EJ Jr, Silberstein LE et al. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1883–94.
18. Neunert C, Lim W, Crowther M et al. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. *Blood*. 2011;117:4190–207.
19. McCrae KR, Samuels P, Schreiber AD. Pregnancy associated thrombocytopenia: pathogenesis and management. *Blood*. 1992;80:2697–714.
20. Aster RH, George JN. Drug-induced thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:776–84.
21. Warkentin T. Thrombocytopenia caused by platelet destruction, hypersplenism, or hemodilution. In Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1895–912.
22. Warkentin TE. Heparin-induced thrombocytopenia. In Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1913–24.
23. Dror Y, Freedman MH. Inherited forms of bone marrow failure. In Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:307–49.
24. Nurden P, Nurden AT. Inherited thrombocytopenias. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:785–95.
25. Sabri S, Foudi A, Boukour S et al. Deficiency in the Wiskott-Aldrich protein induces premature proplatelet formation and platelet production in the bone marrow compartment. *Haematologica*. 2009;94(6):756–59.
26. Greinacher A, Warkentin TE. Acquired nonimmune thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:796–804.
27. Levine SP. Miscellaneous causes of thrombocytopenia. In: Greer JP, Foerster J, Lukens JN et al., eds. *Wintrobe's Clinical Hematology*, 11th ed. Philadelphia: Lippincott Williams & Wilkins; 2004:1565–672.
28. Hoffman R, Kremyanskaya M, Najfeld V, Mascarenhas J. Essential thrombocythemia. In Hoffman R, Benz EJ Jr, Silberstein LE et al. *Hematology Basic Principles and Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1034–52.
29. Bennett JS, Rao AK. Inherited disorders of platelet function. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:805–19.
30. Bernard J, Soulier JP. Sur une nouvelle varieté de dystrophie thrombocytaire hémorragipare congénitale. *Sem Hôp Paris*. 1948; 24:2317.
31. Lopez JA, Andrew RK, Afshar-Karghan V et al. Bernard-Soulier syndrome. *Blood*. 1998;91:4397–418.
32. Smyth SS, Whiteheart S, Italiano JE et al. Platelet morphology, biochemistry, and function. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1735–814.
33. Glanzmann E. Hereditare hamorrhagische thrombasthenie: Ein Beitrag zur Pathologie der Blutplattchen. *J Kinderkranken*. 1918;88:113.
34. Nurden P, Nurden AT. Congenital disorders associated with platelet dysfunctions. *Thromb Haemost*. 2008;99(2):253–63.
35. Sebastiano C, Bromberg M, Breen K et al. Glanzmann's thrombasthenia: report of a case and review of the literature. *Int J Clin Exp Pathol*. 2010;3:443–47.
36. Bellucci S, Damaj G, Boval B et al. Bone marrow transplantation in severe Glanzmann's thrombasthenia with antiplatelet alloimmunization. *Bone Marrow Transplant*. 2000;25:327–30.
37. Franchini M, Lippi G. Recombinant activated factor VII: mechanisms of action and current indications. *Semin Thromb Hemost*. 2010; 36(5):485–92.
38. Albers CA, Cvejic A, Favier R et al. Exome sequencing identifies *NBEAL2* as the causative gene for gray platelet syndrome. *Nat Genet*. 2011;43:735–37.
39. Rao AK, Jalagadugula G, Sun L. Inherited defects in platelet signaling mechanisms. *Semin Throm Hemost*. 2004;30:525–35.
40. Zwaal RF, Comfurius P, Bevers EM. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. *Biochem Biophys Acta*. 2004;1636:119–28.
41. Suzuki J, Umeda M, Sims PJ et al. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature*. 2010;468:834–38.
42. Rao AK, Bennett JS. Acquired disorders of platelet function. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis*: *Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:820–28.
43. Rinder HM. Platelet function testing by flow cytometry. *Clin Lab Sci*. 1998;11:365–72.



34 Disorders of Secondary Hemostasis

ALI GABALI, MD, PhD
BEVERLY KIRBY, EdD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *deficiency* as it relates to the proteins of secondary hemostasis.
2. Describe the expected results of laboratory screening tests that detect abnormalities of the proteins of secondary hemostasis.
3. Describe the expected laboratory results, pathophysiology, and clinical symptoms in patients with classic (type I) von Willebrand disease.
4. Identify hemostatic proteins that are deficient in hemophilias A and B.
5. Characterize classic (type 1) von Willebrand disease and deficiencies of factors VIII and IX by inheritance pattern, clinical symptoms, and laboratory findings.
6. Identify clinical conditions associated with acquired disorders of the hemostatic proteins, and describe the expected results in laboratory screening tests for hemostasis.
7. Characterize disseminated intravascular coagulation (DIC) by etiology, pathophysiology, and the results of laboratory testing.
8. Explain physiologic variations in the reference intervals of laboratory hemostatic screening tests in newborns.
9. Describe the expected general clinical consequences in a patient with an abnormality of the proteins of secondary hemostasis.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Explain the clinical symptoms characteristic of deficiencies of the fibrin-forming proteins, and contrast them with the symptoms associated with disorders of primary hemostasis.
2. Compare and contrast the results of laboratory tests for hemostasis in disorders of primary and secondary hemostasis.
3. Describe genetic mutations and diagram the inheritance pattern that results in deficiencies of the hemostatic proteins.
4. Differentiate von Willebrand disease subtypes, deficiencies of factors VIII and IX, and the inherited deficiencies of the remaining proteins of

(continued)

Chapter Outline

Objectives—Level II *(continued)*

fibrin formation by inheritance pattern, pathophysiology, clinical symptoms, and laboratory findings.

5. Select and interpret the results of laboratory tests, and identify clinical symptoms that differentiate von Willebrand disease subtypes, deficiencies of factors VIII and IX, Bernard-Soulier disease, and Glanzmann's thrombasthenia.
6. Describe the pathophysiology of the conditions that result in acquired abnormalities of the hemostatic system, and select confirmatory laboratory procedures.
7. Select and describe the laboratory screening methods for distinguishing between deficiencies and inhibitors of hemostatic proteins.
8. Describe the significance and clinical implications of the development of circulating anticoagulants, and select laboratory procedures that confirm and differentiate between specific and nonspecific factor inhibitors.
9. Choose laboratory methods that differentiate between excessive primary and secondary fibrinolysis, and support your selection.

Key Terms

Acquired inhibitor (circulating anticoagulant)
Afibrinogenemia
Consumption coagulopathy
Cross-reacting material positive (CRM+)
Cross-reacting material negative (CRM−)
Cross-reacting material reduced (CRM^R)
Disseminated intravascular coagulation (DIC)
Dysfibrinogenemia
Hemophilia A
Hemophilia B
Hemorrhagic disease of the newborn (HDN)
Hypofibrinogenemia
Lupus anticoagulant (LA)
Platelet-type-pseudo-VWD
Primary fibrinogenolysis
Ristocetin-induced platelet agglutination (RIPA)
VWF multimer
Vitamin K deficiency bleeding (VKDB)
von Willebrand disease (VWD)
von Willebrand factor antigen (VWF:Ag)
von Willebrand factor ristocetin cofactor activity (VWF:RCo)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Secondary hemostasis: Describe the formation of fibrin and the process of fibrinolysis. (Chapter 32)
- Laboratory testing in coagulation: Review coagulation screening tests. (Chapter 36)

Level II

- Molecular genetics: Summarize the principles and the uses of molecular diagnostic tests. (Chapter 42)

CASE STUDY

We will address this case throughout the chapter.

Scott, a 2-year-old male, developed a severe bleed into a knee joint and was seen in the emergency room of his local hospital.

Consider which of the three physiologic compartments involved in hemostasis could be responsible for his bleeding and how this diagnosis could be established.

OVERVIEW

This chapter discusses disorders of clotting factors that result in excessive bleeding. Deficiencies of most of the fibrin-forming proteins are included in this category as are some proteins associated with the fibrinolytic system. The pathophysiologic basis and clinical manifestations for each defect are presented. A major emphasis is on the laboratory involvement in the diagnosis and treatment of the defects. Because the levels of coagulation proteins are different in neonates when compared with adults and testing presents a unique challenge, a section on newborn hemostasis also is included.

INTRODUCTION

The process of secondary hemostasis (Chapter 32) results in the formation of fibrin, which stabilizes the primary hemostatic plug. Production of an effective stabilized fibrin clot requires the interaction of several plasma proteins (procoagulant proteins) that become activated by the injured tissue in what has classically been described as a *cascade* sequence. In addition, other proteins are required to inhibit or inactivate the procoagulant proteins so that clot formation is limited to the injured area. Fibrin subsequently is broken down, or lysed, as the wound heals.

If one or more of these proteins are defective, either quantitatively or qualitatively, the balance between clot formation and clot lysis is upset. Fibrin formation is impaired if the fibrin-forming proteins are faulty. If proteins of the fibrinolytic system malfunction, either too much fibrin is formed or lysis of fibrin is inadequate or excessive. The results of such imbalances in the system are symptoms of either excessive bleeding or inappropriate and excessive clotting.

This chapter discusses disorders of the plasma proteins that result in excessive bleeding. Plasma protein disorders resulting in excessive clotting (thrombosis) are discussed in Chapter 35.

DISORDERS OF THE PROTEINS OF FIBRIN FORMATION

Disorders of the proteins of fibrin formation arise either by inheritance of a defective gene that directs the synthesis of a hemostatic protein or by acquisition of a deficiency secondary to another condition during the individual's lifetime. In the hereditary disorders, the genetic defect causes either the failure of synthesis of one of the proteins or the production of a malfunctioning molecule (Web Figure 34-1). In both situations, the rate of fibrin formation is slowed and ineffective, and the patient can experience abnormal bleeding.

Early investigators of coagulation disorders assumed that if a patient bled excessively, a coagulation protein was absent or decreased in quantity and called the defect a *deficiency*. The word *deficiency* currently is applied to either hereditary or acquired disorders and is understood to refer to either the absence of a coagulation protein (quantitative disorder) or to a protein that is present in the plasma but that is functionally defective (qualitative disorder). The defect affects fibrin formation both in vivo and in vitro in laboratory screening tests such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT) (Chapter 36).

Most of the laboratory screening tests are clot based and measure, in seconds, the length of time that it takes a fibrin clot to form in plasma. These tests depend, therefore, on the presence of an adequate amount of the clot-forming proteins (quantity) and how well the proteins function (quality). Generally, clot-based tests do not differentiate qualitative from quantitative defects. A functionally defective coagulation factor prolongs the clotting screening test but can still be recognized by immunologically based procedures in the laboratory, which utilize antibodies to detect the presence of the protein. Individuals who have these functionally defective factors that can be detected immunologically are said to be **cross-reacting material positive (CRM+)**. Patients in whom the clotting factor is quantitatively decreased (absent) by both functional and immunological assays are **cross-reacting material negative (CRM−)**. Finally, there are patients in whom both functional and immunological assays are equally reduced, but measurable protein/activity is present. These individuals are described as **cross-reacting material reduced (CRM^R)** (Web Figure 34-1).

Clinical bleeding symptoms in patients with coagulation factor deficiencies differ from those seen in platelet defects (Table 34-1 ★). These patients bleed from the rupture of small arterioles rather than from capillaries, and the sites of bleeds are into deep muscular tissues and joints rather than the superficial areas seen in platelet disorders. Hematomas are common and can be massive. Patients also experience delayed bleeding from cuts. Patients with coagulation factor defects usually have normal platelets; therefore, a typical primary hemostatic plug is formed after a superficial cut that initially arrests the blood flow, and the bleeding stops. Delayed bleeding occurs because in the absence of stabilization with fibrin formation, the plug dislodges and the wound begins to bleed again later. The subsequent bleed usually continues for a longer time with the loss of a larger amount of blood.

★ **TABLE 34-1** Bleeding Characteristics in Disorders of Secondary Hemostasis

Symptoms typical of secondary hemostatic disorders

- Delayed bleeding
- Deep muscular bleeding
- Joint bleeding

Symptoms common to primary and secondary hemostatic disorders

- Ecchymoses
- Gastrointestinal bleeding
- Hematuria
- Hypermenorrhea
- Gingival (gums) bleeding
- Increased bleeding after tooth extraction
- Intracranial bleeding
- Epistaxis

Patients with coagulation factor deficiencies can experience ecchymoses, excessive bleeding from traumatic injuries, and bleeding from the body sites listed in Table 34-1. Patients with platelet disorders also can have some of these symptoms. Retroperitoneal bleeding and hematuria are common, but petechiae are not usually seen in disorders of secondary hemostasis.

The physical evaluation and laboratory investigation of a bleeding patient proceed as described in Chapter 33. The battery of screening tests in a factor deficiency usually shows a normal platelet count, but the PT, the APTT, or both are usually prolonged (Table 34-2 ★). In the case of von Willebrand disease (VWD), at times the only abnormal laboratory test may be the bleeding time, platelet aggregation studies, or platelet function analysis by PFA-100®. The patient can also have only a strong family or personal history of bleeding with no abnormal laboratory results (see the section "Laboratory Tests for von Willebrand Factor [VWF]" Chapter 36). On the other hand, the thrombin clotting time may be the only abnormal test in disorders of fibrinogen. Abnormalities of factor XIII (FXIII) require specific testing. When the history and results of the screening tests indicate, additional testing should be ordered to specifically define the problem. Coagulation factor assays, fibrinogen levels, a D-dimer test, and/or antithrombin levels can be useful confirmatory procedures (Chapter 36).

This chapter discusses the hereditary disorders of each of the coagulation factors on the basis of inheritance pattern. The most common types of VWD demonstrate an autosomal dominant pattern of inheritance; hemophilias A and B show X-linked recessive inheritance. Deficiencies of the remaining clotting factors exhibit an autosomal recessive inheritance pattern. Acquired disorders of the clotting factors follow and are classified as consumption disorders, liver disease, vitamin K deficiencies, and acquired pathologic inhibitors.

The amino acid sequences of most of the hemostatic proteins and their inhibitors have been determined as well as the nucleotide sequences of most of their genes. Research laboratories are characterizing the molecular sites and types of mutations that lead to coagulation factor deficiencies. Specific molecular defects in many genes and their corresponding proteins have been established and include point mutations, alterations of splice junctions, deletions, insertions, inversions, and mutations resulting in premature stop codons. Researchers are attempting to correlate sites and types of mutations with the clinical symptoms seen in patients.

★ **TABLE 34-2** Coagulation Screening Test Results in Congenital Deficiencies

Platelet Count	PT	APTT	PFA-100	TT	Suspected Congenital Deficiency
N	N	N	N	N	Factor XIII, mild deficiencies of any factor, α_2-antiplasmin, plasminogen activator inhibitor-1
N	A	N	N	N	Factor VII
N	N	A	N	N	Factors XII, XI, IX, VIII, prekallikrein, high molecular weight kininogen
N	A	A	N	N	Factors X, V, II (prothrombin)
N	A	A	N	A	Fibrinogen
N	N	A or N	A or N	N	von Willebrand disease

N = normal; A = abnormal; PT = prothrombin time; APTT = activated partical thromboplastin time; TT = thrombin time

Hereditary Disorders of Secondary Hemostasis

Secondary hemostasis disorders can be inherited as autosomal dominant, X-linked recessive, and autosomal recessive disorders. Inherited coagulation factor disorders usually involve a single coagulation protein, and if bleeding occurs, it is generally from a single site.

Autosomal Dominant Inheritance

Two coagulation disorders exhibit an autosomal dominant pattern of inheritance: von Willebrand disease (types 1, 2A, 2B, 2M) and dysfibrinogenemia. Dysfibrinogenemia will be discussed with the other disorders of fibrinogen in the section "Autosomal Recessive Disorders." von Willebrand disease is considered here.

von Willebrand Disease

von Willebrand disease (VWD) is a quantitative or qualitative deficiency of von Willebrand factor (VWF) arising from mutations in the VWF gene. Dr. Erik von Willebrand first described the disease that carries his name after studying an extended family in the Åland Islands of Finland in 1926.[1] The first patient studied died during her fourth menstrual period, and four sisters also had died from bleeding. At the time, available laboratory tests included the platelet count, coagulation time, and clot retraction, all of which were normal. With further studies, von Willebrand concluded that the disease was related to platelet dysfunction.

Chapter 31 described the role of VWF in primary hemostasis. VWF serves as a bridge between GPIb/IX receptors on activated platelets and subendothelial collagen exposed when the vessel is injured (primary hemostatic plug formation).[2] To perform these activities, VWF subunits must assemble into large multimers. VWF also plays a role in secondary hemostasis, complexing with and stabilizing circulating coagulation FVIII in the plasma (Chapter 32). The function of VWF in both primary hemostasis and fibrin formation is altered in most patients with clinically symptomatic VWD.

Inheritance Characteristics of VWD. Inherited defects in VWF can result in defective protein processing or disruption of specific ligand-binding sites. Based on the nature of the mutation and inheritance patterns, VWD is subdivided into three types. Type 1 and type 3 VWD are quantitative deficiencies, whereas type 2, consisting of four subtypes, are qualitative deficiencies. VWF is a carrier protein for FVIII and in most cases when the levels of VWF decrease, FVIII decreases in parallel. Therefore, the results of laboratory tests in VWD can sometimes be confused with a deficiency of FVIII. The similarities and differences between VWD and FVIII deficiency will be addressed later.

The autosomal dominant pattern of inheritance produces clinical symptoms when one gene is defective (heterozygous) and the production of VWF is reduced by about one-half. Circulating levels of FVIII generally are reduced in proportion to the reduction in VWF. The platelet-associated function of VWF is independent of the presence or absence of FVIII. Although the inheritance pattern of most patients with VWD is autosomal dominant, more severely affected patients (with types 2N and 3 disease) demonstrate an autosomal recessive or compound heterozygous pattern. Symptomatic patients with these disorders have mutations in both the VWF genes (i.e., are homozygotes or double heterozygotes).

Synthesis and Structure of von Willebrand Factor. Figure 34-1 ■ depicts the synthesis of the FVIII/VWF complex. The structure of the mature VWF molecule is a multimeric chain of identical subunits. Normally, the number of subunits in individual molecules varies so that the molecular weights range from 0.5 to >20 million daltons (Da). Although VWF in plasma consists of a wide range of sizes, all VWF molecules bind FVIII in a 1:1 molar ratio (Chapter 32). A detailed description of the synthesis of VWF, which occurs in endothelial cells and megakaryocytes, can be found in this text's Companion Resources.

A schematic diagram of the structure of a VWF subunit (Figure 34-2 ■) consists of four types of domains, A through D, which are repeated and numbered in the arrangement shown. To perform its functions in both primary and secondary hemostasis, the mature VWF multimer must interact with a number of ligands. Sites of interaction with FVIII, GPIb/IX, GPIIb/IIIa, collagen, and heparin have been identified.[2,3] The diagram also includes the major sites of mutations in the various subtypes of type 2 VWD.

Clinical Findings. VWD is the most common inherited bleeding disorder. It is estimated that about 125 in 1 million persons have VWD with clinical (bleeding) symptoms and another 1% of the population is asymptomatic and thus not aware of having the disease.[4]

Bleeding symptoms, if present, are usually mild. Those patients who are homozygous or doubly heterozygous for VWD can exhibit severe symptoms and have the potential for life-threatening bleeding. Typical bleeding includes hemorrhage in mucosal and cutaneous tissues: ~60% of patients report epistaxis, 50% report menorrhagia and bleeding after dental extraction, and 35% report gingival bleeding and

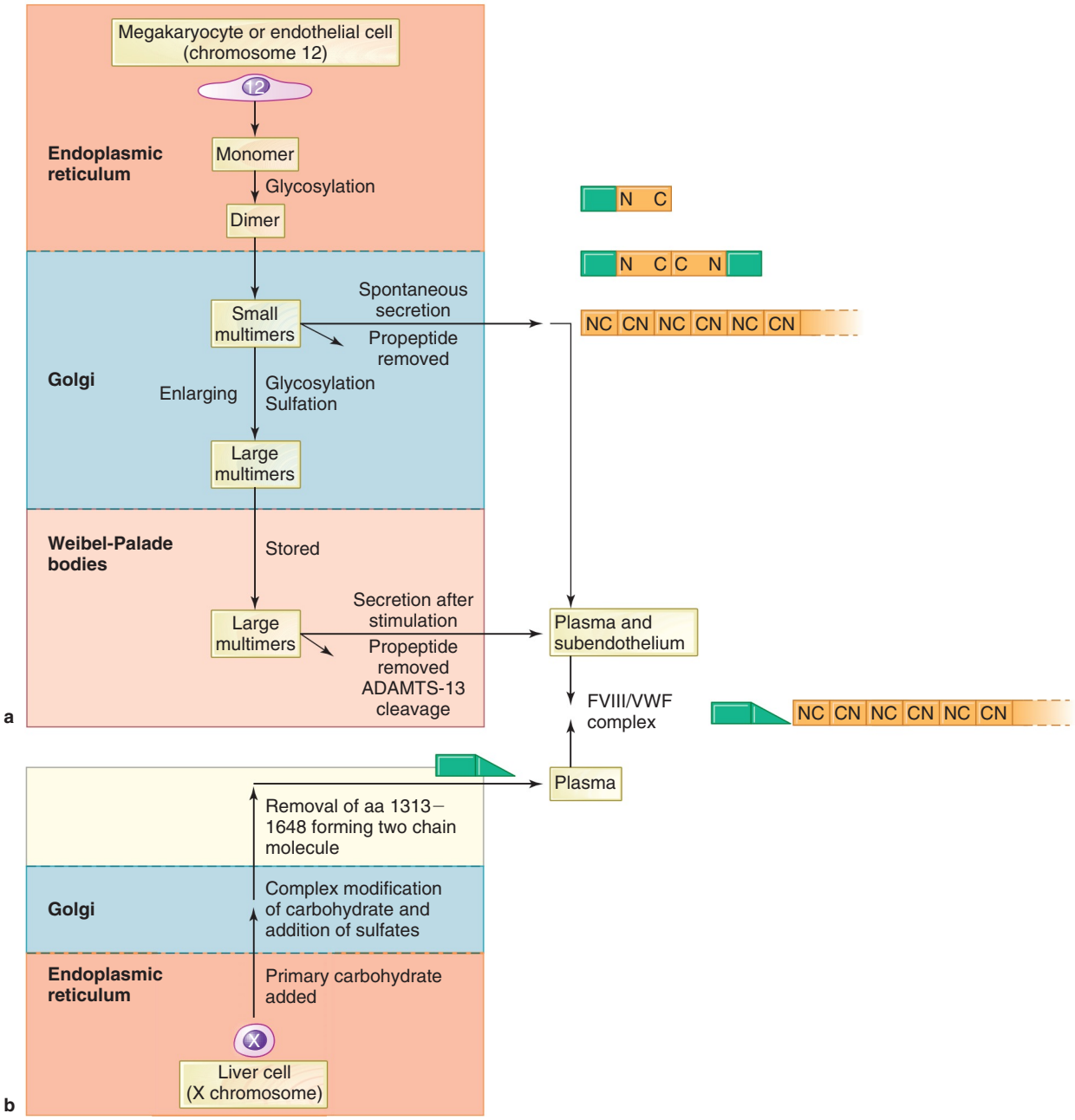


FIGURE 34-1 Synthesis and secretion of FVIII/von Willebrand complex. **(a)** Synthesis of von Willebrand factor. VWF transcripts are processed in the endoplasmic reticulum (ER) and then the Golgi. Subunits of VWF are synthesized in the ER as single-chain proprotein monomers. Dimers are formed by attachment of two monomers at the carboxyterminal (C) ends. Dimers move to the Golgi where the propeptide is removed and the dimers associate at the amino terminal (N) ends to form large multimers. **(b)** Synthesis of FVIII in the liver. Both VWF and FVIII are secreted into the plasma where they bind together. = propeptide; = FVIII

easy bruising.[5,6] These clinical features resemble those seen in platelet disorders. Excessive bleeding at childbirth is comparatively rare because in pregnancy, the activity of the entire FVIII/VWF complex increases, but postpartum bleeding has been reported to occur in 21–59% of patients.[7] The symptoms in individuals with type 3 VWD (homozygous) can include hemarthroses and spontaneous deep tissue bleeding resembling coagulation factor deficiencies because of the concomitant decrease in FVIII levels. An individual's ABO blood type affects VWF antigen levels (see the section "Laboratory Evaluation of VWD"), which may affect the severity of bleeding symptoms although the impact is still somewhat controversial.[8,9]

One hallmark of VWD is its phenotypic variability. The severity of bleeding symptoms differs in individuals within the same kindred, among kindreds, and, from time to time, within the same individual.

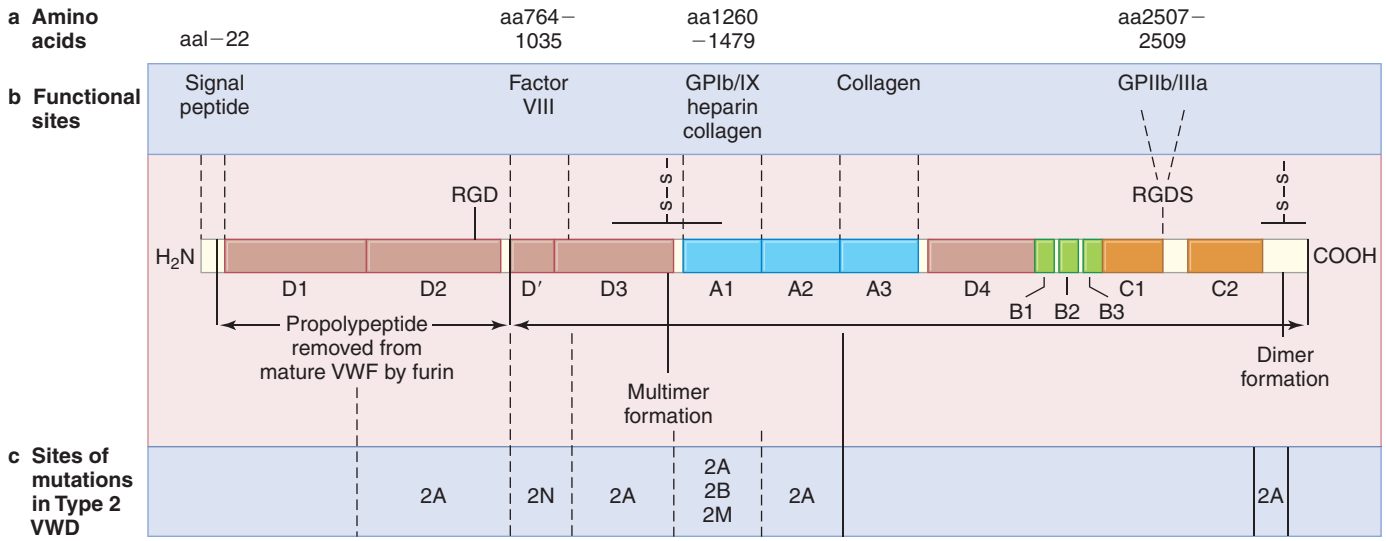


FIGURE 34-2 A diagrammatic depiction of the domain structure of a VWF monomer precursor protein. The precursor protein consists of 2813 amino acids. **(a)** The amino acids (aa) involved in the functions at **(b)** aa 1–22 is the signal peptide; domains D1 and D2 (aa 23–741) are the propeptide removed during processing; domain D′ (aa 764–1035) plus part of domain D3 are the binding site for FVIII; domain A1 (aa 1260–1479) contains binding sites for GPIb, heparin, and collagen; domain A3 contains the major binding site for collagen; domain C1 contains the binding site for GPIIb/IIIa; disulfide bridges at D3 and the C terminal end are the sites of attachment when forming multimers and dimers, respectively. The mature protein with the propeptide removed contains 2050 amino acids. **(c)** Sites of mutations of the subtypes of type 2 VWD.

R = arginine; G = glycine; D = aspartic acid; S = serine

Symptoms may not begin until the second decade of life in mild forms of the disease. In the severe forms, symptoms begin early in life and often decrease with age. Laboratory test results vary, often requiring repeated testing of the same individual (Chapter 36).[3] The severity of clinical symptoms does not necessarily correlate with the level of VWF activity. However, some VWD subtypes demonstrate unique symptoms related to the nature of the mutations.

Laboratory Evaluation of VWD. The common laboratory tests for fibrin formation do not directly evaluate VWF. The laboratory diagnosis of VWD is based on the results of a battery of tests after identification of a patient with a lifelong clinical history of the typical bleeding symptoms and/or a history of similar symptoms in other family members. Screening tests include the platelet count, APTT, PT, the template bleeding time (BT), and/or the PFA-100®. The platelet function analyzer (PFA-100), a device that uses a membrane coated with collagen and a platelet agonist to which platelets adhere, has replaced the bleeding time in most institutions.[10] Typical results of these tests in VWD are shown in Table 34-3 ★ and are compared with the results seen in the hemophilias (see "X-Linked Recessive Disorders").

The BT is abnormal not because of an intrinsic platelet abnormality but because in the absence of VWF, the platelets are unable to adhere to collagen and initiate the formation of the primary hemostatic plug (Chapter 31; Figure 33-6b). The PFA-100 closure time is abnormal with collagen/ADP and collagen/epinephrine cartridges for similar reasons.[9,11] APTT is used as an indirect screening test for VWF because of its correlation with FVIII levels. An abnormal APTT occurs only when the level of FVIII is near or below the APTT reagent's sensitivity (for most commercial reagents FVIII level ≤30% of normal). Therefore, BT, PFA, and APTT results can produce discordant normal/abnormal results (Chapter 36). The PT, which does not depend on either FVIII or VWF, is normal.

If the screening test results and the patient's clinical history suggest VWD, specific tests are required to establish the diagnosis and to determine the type or subtype of VWD. These tests quantitate VWF and FVIII activity and determine various functional and structural aspects of the VWF protein (Chapter 36).

VWF cannot be measured by clot-based assays; thus, immunologic tests are used to quantitate the amount of VWF protein in the plasma as **von Willebrand factor antigen (VWF:Ag)**. Various immunologic methodologies (e.g., ELISA) are available and use commercially prepared monoclonal antibodies to VWF. FVIII activity is determined by a standard factor assay method and usually correlates with the amount of VWF antigen (Chapter 36). The patient's ABO blood type should also be determined because blood type significantly affects the level of VWF protein (Table 36-6). Individuals with blood group O have 25–30% less VWF protein than individuals with A or B antigens. This is thought to be caused by an effect on carbohydrate structure and VWF clearance from plasma. VWF is a glycoprotein that carries the A, B, and H carbohydrate structures. The lack of the A and B carbohydrates on circulating VWF is believed to result in a more rapid hepatic clearance of the protein. Some laboratories have developed

★ **TABLE 34-3** Laboratory Evaluation of von Willebrand Disease (VWD) and the X-Linked Recessive Disorders

	VWD, Type 1	Factor VIII Deficiency	Factor IX Deficiency
Platelet tests			
Platelet count	N	N	N
Template bleeding time	N/Increased	N	N
Platelet function analyzer	N/Increased	N	N
Platelet aggregation test			
ADP	N	N	N
Collagen	N	N	N
Epinephrine	N	N	N
Ristocetin	Decreased/Absent[a]	N	N
Coagulation factor tests			
Prothrombin time	N	N	N
Activated partial thromboplastin time	N or increased	Increased	Increased
Thrombin time	N	N	N
Factor VIII assay	N/Decreased	Decreased	N
Factor IX assay	N	N	Decreased
VWF:Ag assay	Decreased	N	N
Fibrinolysis tests	N	N	N

[a]Except for type 2B VWD (in which aggregation with ristocetin is increased). Decreased or absent when VWF:A ≤30%; N = normal

different reference intervals for each blood type. Also, because VWF is an acute phase reactant, levels of protein are increased during inflammatory states, stress, or pregnancy.

The ability of VWF to function in platelet adhesion is determined by the **von Willebrand factor ristocetin cofactor activity (VWF:RCo)** that is performed on a platelet aggregometer and measures the ability of the patient's VWF to support agglutination of platelets by ristocetin (Chapter 36).

The diagnosis of VWD is established by finding a decreased plasma level of VWF activity, VWF antigen, FVIII levels (which usually correlate with the quantity of VWF), and/or a prolonged bleeding time or prolonged PFA-100 closure time. Classic (type 1) VWD results from a partial quantitative deficiency of VWF and typically is associated with abnormal results in all of these tests. In some cases, however, one or more of the tests can be normal, making diagnosis more difficult. Diagnosis of type I VWD is also complicated by the fact that the reference interval for VWF is extremely broad. The functional VWF:RCo activity might not correspond to the VWF antigen levels in some patients, depending on whether they have a quantitative or a qualitative defect. Patients with qualitative defects can have decreased concentrations of VWF antigen but more markedly abnormal functional tests than would be expected by the amount of VWF present.

When the diagnosis of VWD is established by decreased VWF:Ag and/or abnormal function of VWF, the final step in the laboratory diagnosis of VWD is to establish the subtype. The structure of **VWF multimers** is studied by electrophoresis using 1% agarose gels in the presence of sodium dodecyl sulfate (SDS). The multimers are separated by size and visualized as bands. These tests can be performed on platelet- or plasma-derived VWF. Other tests of VWF functional activity include ELISA tests designed to measure the ability of the patient's VWF to bind to either FVIII or to collagen, described as VWF:CB (collagen binding) and VWF:VIIIB (FVIII binding) assays.[12,13] The **ristocetin-induced platelet agglutination test (RIPA)** measures the ability of ristocetin to induce agglutination of patient platelet rich plasma (PRP). Thus, it detects the patient's VWF binding to the patient's platelet GPIb/IX. In patients with rare qualitative mutations, one or more of these abilities is decreased. Theoretically, DNA analysis to establish the specific gene mutation would be desirable, but is difficult because the VWF gene on chromosome 12 is very large and complex with a high degree of polymorphism. Databases for VWD mutations have been established,[9] and an online database is accessible at www.shef.ac.uk/vwf/index.html. Currently, most clinicians believe that DNA testing for VWD should be used to confirm diagnosis rather than screening or initial diagnosis in most instances. DNA testing is used for prenatal diagnosis of severe type 3 VWD. In the future, with improvements in methodology, DNA analysis could become the test of choice for differentiating the qualitative subtypes of VWD type 2 because those mutations are more predictable than the ones resulting in the quantitative types of VWD.[6]

Classification. A classification system for VWD proposed in 1994 simplifies previous schemes and is based on the phenotype of the VWF protein determined using the laboratory tests described previously.[14] This classification was updated in 2006 to include an improved understanding of the metabolism of VWF and has been used since then.[15] This scheme has three major categories, types 1, 2, and 3, which depend on whether the patient has a quantitative or a qualitative defect and, in the case of quantitative defects, the extent of the quantitative deficiency. Types 1 and 3 VWD are both quantitative deficiencies of VWF. Type 1, the most common ("classic") type, is a mild form of VWD in which patients have a partial quantitative

★ TABLE 34-4 Description of von Willebrand Disease (VWD) Subtypes

Type	Description	% of VWD	Mutation Sites; Functional Abnormalities
1	Partial quantitative deficiency with normal structure and function of the multimers	70–80	Multiple
2	Qualitative disorder with functionally abnormal VWF		
2A	Decreased platelet adhesion; absence of largest multimers	10–15	A2, A1, D2, D3 domains—either defective synthesis of largest multimers (low plasma levels of VWFpp[a]) or increased proteolysis (high plasma levels of VWFpp)
2B	Increased affinity for platelet GPIb; absence of largest multimers	<5	A1 domain—spontaneous binding to platelets and removal from circulation
2M	Decreased platelet adhesion not due to absence of largest multimers	Rare	A1 domain—abnormality of platelet ligand-binding sites (GPIbα); A3 domain—abnormality of collagen binding site
2N	Decreased affinity for FVIII (autosomal recessive)	Rare	D′, part of D3 domains—platelet-dependent functions preserved; loss of function as carrier of FVIII (mutations affecting FVIII binding site)
3	Absence of VWF in platelets and plasma	0.5–5 per million	Varied; 64% characterized

[a] VWFpp = VWF propeptide

deficiency of VWF. Type 3 patients have an absolute absence of VWF (a severe form of the disease). Type 2 patients have qualitatively abnormal VWF of various kinds; type 2 is further subdivided into four variants, types 2A, 2B, 2M (M stands for multimers), and 2N (N stands for Normandy).[16] Discrepancies between the level of VWF:Ag and VWF functional assays characterize qualitative disorders.

The structure of the VWF protein with sites of mutations is depicted in Figure 34-2. Characteristics of the VWF types are summarized in Table 34-4 ★. These subtypes are discussed in more detail in this chapter's Companion Resources and in the Companion Resources that accompany Chapter 36. See Table 34-5 ★ for a summary of laboratory test results in all types and subtypes of VWD. Discriminating qualitatively normal and abnormal VWF by routine laboratory testing is difficult. A common approach used by many laboratories is to compare values obtained in functional assays (VWF:RCo, VWF:CB, VWF:FVIIIB) with VWF:Ag. At a ratio of <0.6[12] or <0.7[13] the VWF protein is considered to be abnormal (i.e., dysfunctional).

☑ CHECKPOINT 34-1

a. Why do patients with type 1 VWD have 25–50% of VWF in their plasma?

b. Why do they have a corresponding decrease in FVIII in their plasma?

★ TABLE 34-5 Differentiation of Subtypes of von Willebrand Disease (VWD)[7]

Type	1	3	2A	2B	2M	2N
Mode of inheritance	Autosomal dominant	Homozygous or double heterozygous	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal recessive
Screening tests						
Platelet count	Normal	Normal	Normal	Decreased	Normal	Normal
Bleeding time	Normal or increased	Increased	Increased	Increased	Increased	Normal
Closure time	Normal or increased	Increased	Increased	Increased	Increased	Normal
Diagnostic tests						
VWF:Ag assay	Decreased	Absent	Normal or decreased	Normal or decreased	Normal or decreased	Normal
Factor VIII assay	Normal or decreased	Severely decreased	Normal or decreased	Normal or decreased	Normal or decreased	Decreased
VWF:RCo	Decreased	Absent	Decreased relative to VWF:Ag	Normal to decreased	Decreased relative to VWF:Ag	Normal
Tests to determine subtype						
RIPA	Normal or decreased	Absent	Decreased relative to VWF:Ag	Increased	Decreased relative to VWF:Ag	Normal
Multimer analysis	Normal	Absent	Absence of large and intermediate	Absence of large	Normal	Normal

VWF:Ag = von Willebrand factor:antigen; VWF:RCo = von Willebrand factor:ristocetin cofactor activity; RIPA = ristocetin-induced platelet agglutination

Related Clinical Syndromes. Two clinical syndromes with similar features to VWD are **platelet-type-pseudo-VWD** and acquired von Willebrand syndrome. Although clinical bleeding symptoms and laboratory test results are similar to those in VWD, they are not caused by mutations in the VWF gene.

Pseudo-VWD, a platelet disorder characterized by increased affinity of the platelet GPIb/IX receptor for VWF, is clinically similar to VWD type 2B, but the defect is usually a gain-of-function mutation of GPIbα (rather than VWF), resulting in spontaneous binding of larger multimers of plasma VWF to platelet GPIb. This results in a decrease of large VWF multimers and FVIII in the plasma. In addition, GPIIb/IIIa receptors are exposed and platelets aggregate, resulting in thrombocytopenia.

Acquired VW syndrome (AVWS) is called a *syndrome* rather than a *disease* because it is not caused by VWF mutations. AVWS is rare and has been associated primarily with lymphoproliferative diseases such as lymphoma and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), multiple myeloma, monoclonal gammopathy of unknown significance (MGUS), and autoimmune diseases. The pathogenic mechanism of AVWS varies from patient to patient, although most are associated with loss of VWF because of neutralizing anti-VWF antibodies (autoimmune clearance), proteolytic or mechanical degradation of the protein, or adsorption to cell surfaces. AVWS is diagnosed with the same laboratory tests used for inherited VWD: FVIII coagulant activity, VWF:Ag, and VWF:RCo.[17] Elevated plasma levels of the VWF propeptide (VWFpp) can sometimes help distinguish between acquired and congenital disease (VWFpp is normal or elevated in AVWS but decreased in most types of VWD). However, this result is not completely specific for acquired VWS because some forms of congenital disease also are associated with increased clearance of VWF.[18]

Prenatal Diagnosis. Prenatal diagnosis of VWD is available but is usually reserved for patients with severe types of the disease. Type 2 is easily identified prenatally by polymerase chain reaction (PCR) techniques, but diagnosis of type 3 is more difficult because the known mutations account for only a small subset of such patients.[4]

VWD Therapy. Patients with mild VWD who are not experiencing clinical bleeding do not require therapy. Several preparations are available for VWD patients who are actively bleeding to raise the level of VWF in their plasma. The classic treatment for VWD was cryoprecipitate preparations containing all molecular forms of VWF including the large multimer forms, as well as FVIII and fibrinogen. A drawback to using cryoprecipitate is that it cannot be treated to inactivate blood-borne viruses and, therefore, carries a risk of transmission of blood-borne disease including HIV and hepatitis viruses.

The current preferred method of treatment (if effective) is the modified antidiuretic hormone, deamino-D-arginine vasopressin (DDAVP), which induces endothelial cell release of VWF from the Weibel-Palade bodies. DDAVP temporarily increases the levels of VWF and FVIII in some patients and can be used to treat patients who are bleeding. Transmission of blood-borne disease is not a risk with DDAVP. About 20–25% of VWD patients, including all type 3 patients and about 10–15% of type 1 patients, do not respond to DDAVP. The response can be variable in type 2 patients, depending on subtype. It should be used with caution to treat patients with type 2B VWD because it can cause a dangerous reduction in the platelet count. Patients with type 2A do not respond consistently.

The preferred form of therapy for those who do not respond to DDAVP currently is concentrated "intermediate purity" preparations of FVIII that contain intermediate-size VWF molecules. High-purity or monoclonal FVIII preparations do not contain sufficient VWF to be useful in treating VWD. FVIII concentrates are virus inactivated by a variety of methods. A potential complication of using products containing both FVIII and VWF is an elevation of plasma FVIII levels, sometimes to as high as 400 IU/mL. Excessively high levels of FVIII can be associated with venous thrombosis (Chapter 35). A highly purified VWF concentrate that contains little FVIII (Wilfactin®) has been used successfully in Europe but is not yet available in the United States.[19]

☑ CHECKPOINT 34-2

A patient with VWD has an equal decrease in FVIII activity, VWF:RCo, and VWF:Ag assay.

a. Does this more likely indicate that there is a true decrease in the amount of VWF, or is it more likely to indicate that the patient has a type of VWD that is characterized by a functional abnormality of VWF? Support your answer.

b. What type of VWD is most likely in a patient with these laboratory results?

CASE STUDY (continued from page 690)

Scott's mother was questioned about how the bleeding had begun in the joint and about other bleeding history. She answered that Scott had taken a slight tumble, but that it had not seemed to her enough to cause such severe bleeding. She also mentioned that when Scott had minor cuts, they seemed to stop bleeding quickly but often would bleed again in a day or two.

1. What term is used to describe the type of bleeding from minor cuts that Scott's mother is describing?
2. Does this history seem to be typical of a platelet disorder or of a coagulation factor disorder? Why?

X-Linked Recessive Disorders

The hemophilias are hereditary bleeding disorders resulting from congenital deficiencies of proteins involved in blood coagulation. The clinically most common deficiencies are associated with mutations in the genes for factors VIII (*F8*) and IX (*F9*) on the X chromosome. These proteins participate as a cofactor (FVIII) and serine protease (FIX) in the intrinsic pathway of fibrin formation (Chapter 32). Deficiency of FVIII is known as **hemophilia A**; deficiency of FIX is called **hemophilia B** (also known as *Christmas disease*). Both deficiencies demonstrate X-linked recessive inheritance patterns.

Historical Background

Hemophilia has been known for several thousand years. It has contributed to world history by its presence in the royal families of Europe, particularly Great Britain, Russia, and Spain through Queen Victoria who was a carrier for hemophilia. It had been assumed that Queen Victoria had been a carrier of hemophilia A because of the relative prevalence of FVIII versus FIX deficiency. In 2009, the bones of some members of the Imperial Russian family were recovered, and genomic analysis revealed that, in fact, she had passed the gene for hemophilia B throughout the royal families of Europe.[20]

Originally, all patients with X-linked bleeding disorders were believed to have the same disease (deficiency of FVIII). This idea was challenged when in 1947, Pavlovsky observed that prolonged recalcification times (the test available then) on two patients with hemophilia were corrected when the test was performed on mixtures of the two plasmas.[21] In 1952, three groups of investigators reported patients who were missing a new clotting factor that became known as FIX.

Inheritance Characteristics

X-linked recessive disorders are usually inherited by sons from their carrier (heterozygous) mothers who have an abnormal allele on one X chromosome and a normal allele on the other. Each son has a 50% chance of inheriting the affected gene. Males have only one X chromosome (hemizygous); thus, if they inherit an abnormal allele on that chromosome, they are affected with hemophilia. Males synthesize variable amounts of the clotting factor, depending on the particular mutation inherited.

Hemophilia A accounts for 80–85% of all cases of hemophilia with a prevalence of approximately 1 in 5,000–10,000 male births. Most of the remaining 15–20% of hemophilia patients are deficient in FIX (affecting ~1 in 30,000 males). Hemophilia A is second to VWD in the overall frequency of inherited bleeding disorders. Approximately 30% of the affected individuals have no positive family history of the disease, indicating that de novo genetic mutations occur often.

Pathophysiology of Bleeding in the Hemophilias

Hemophilia A is a deficiency of the FVIII portion of the FVIII/VWF complex as opposed to VWD in which the VWF portion of the complex is abnormal (discussed previously). Patients with hemophilia A have normal circulating levels and functionally normal VWF. Thus, their platelets adhere properly to collagen, and the formation of an effective primary hemostatic plug is not disrupted. Patients with hemophilia B have an intact FVIII/VWF complex and likewise have normal primary hemostatic plug formation. The abnormal bleeding in both hemophilia A and B is caused by inadequate thrombin formation, delayed and inadequate fibrin formation, a secondary increase in fibrinolysis, and failure to form a stable hemostatic plug at sites of vascular damage. These patients generate a normal tissue factor–driven *initiation* phase of coagulation but fail to generate the massive amounts of thrombin during the *propagation* phase of coagulation (Chapter 32). As a result, in addition to inadequate thrombin and fibrin production, thrombin activation of thrombin-activatable fibrinolysis inhibitor (TAFI) fails to occur, resulting in excessive fibrinolysis and compounding the bleeding associated with inadequate fibrin formation.

Factor VIII Nomenclature

In 1985, the International Committee on Thrombosis and Hemostasis published recommendations that defined nomenclature for the FVIII/VWF complex.[22] Before these recommendations, the literature referring to FVIII and VWF was quite confusing. The currently accepted definitions and abbreviations for the FVIII/VWF complex components are shown in Table 34-6 ★.[22] The original name for FVIII, *antihemophilic factor*, is still an acceptable synonym.

☑ CHECKPOINT 34-3

What abbreviation is acceptable for:

a. the antigenic properties of VWF?

b. the functional activity of FVIII?

c. the complex of FVIII and VWF?

FVIII Mutations

Refer to Figure 34-3 ■ for the molecular structure and key sites of functional activities of FVIII.[23] Genetic defects in the *F8* gene cause hemophilia A. Using restriction enzyme techniques, the polymerase-chain reaction, and other methods, scientists have determined the molecular defects in >4500 patients. The Hemophilia A Mutation Database (www.hadb.org.uk) lists >2100 different mutations. Genetic defects include point mutations (>50% of the identified defects), gross deletions, and regulatory defects spread throughout the gene.[24,25] Mutations result in either quantitative or qualitative defects of the FVIII protein. The majority of mutations result in a CRM− or CRM^R phenotype (~95%). The clinical severity of the disease depends on the site of mutation within the gene and the molecular functions of the protein that are disrupted. One unique type of mutation involving intron 22, called the *FVIII inversion mutation*, occurs in almost 50% of patients with a severe phenotype. The same mutation occurs in all members of a family, resulting in a similar clinical expression of disease in affected family members.

★ **TABLE 34-6** Nomenclature of the FVIII/von Willebrand Factor Complex

von Willebrand factor—deficient in von Willebrand disease (VWD)	
VWF	von Willebrand factor protein
VWF:AG	Antigenic properties of von Willebrand factor as measured by monoclonal antibodies with immunologic procedures
VWF:RCo	Functional activity of the VWF molecule as measured by ristocetin cofactor activity
Factor VIII—deficient in hemophilia A	
FVIII	FVIII protein
FVIII:C	Functional activity of FVIII as a procoagulant in fibrin formation as measured by APTT
FVIII:Ag	Antigenic properties of FVIII as measured by immunologic procedures

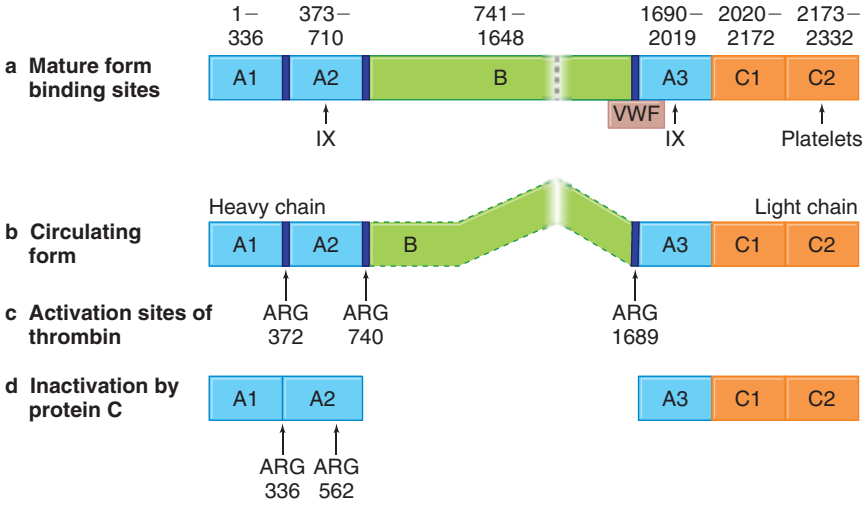


FIGURE 34-3 Schematic diagram depicting FVIII. **(a)** The mature protein with domains A1, A2, B, A3, C1, and C2. **(b)** The circulating form of FVIII with variable portions of the B domain removed; attaches to VWF at the acidic region preceding domain A3. **(c)** Sites of activation by thrombin at Arg residues 372, 740, and 1689. The heavy chain is cleaved, forming a 3-chain structure, and FVIII is released from VWF. **(d)** Sites of proteolytic inactivation of FVIII by activated protein C (APC) at Arg 336 and Arg 562.

ARG = arginine

Factor IX Mutations

FIX deficiency, or hemophilia B, is also known as *Christmas disease* (*Christmas* was the surname of the first reported affected family). Heterogeneous mutations in the FIX gene or its regulatory components result in hemophilia B. A database of mutations, available at www.factorix.org, includes >1100 unique mutations. Point mutations (mis-sense and nonsense), deletions of various portions of the gene, and insertions are the main types of molecular events.[25] The clinical severity depends on the type of mutation and the region of the gene affected. Some FIX deficient patients are CRM−, approximately one-third are CRM+, and some have reduced levels of antigen (CRM^R).

Clinical Aspects of the Hemophilias

The clinical presentations in both hemophilia A and B are identical. They can be distinguished only by laboratory testing. Clinical manifestations vary with the amount of factor present and are classified as severe, moderate, or mild disease (Table 34-7 ★). In general, individuals with >30% activity do not have abnormal bleeding complications. The variation in clinical symptoms is largely the result of the type and site of the mutation (e.g., FVIII-deficient patients who have mutations at the thrombin-cleavage sites of FVIII are unable to activate FVIII to FVIIIa, and patients with the inversion mutation have no FVIII activity and severe bleeding symptoms).

Clinical symptoms in severe disease can begin at circumcision. Hemarthrosis is the most common feature of severe hemophilia. Bleeding into a joint can be triggered by even minor trauma and is accompanied by intense pain. The joint fills with blood, some of which is not reabsorbed, causing chronic inflammation, pain, and eventually joint destruction. Joint bleeds, particularly into the knee and ankle, generally occur when a child starts to walk. Subcutaneous hematomas can begin with slight trauma and spread to involve a large mass of tissue, causing purple discoloration of the skin. Epistaxis is rare in hemophilia. Other manifestations include hematuria, deep muscle bleeding, excessive bleeding from dental extractions, bleeding with intramuscular injections, and delayed bleeding after minor cuts. The most common cause of death (after exclusion of viral infections transmitted by the replacement product) is intracranial hemorrhage, which can occur spontaneously or after trauma.

Hemarthrosis and severe, spontaneous, crippling bleeding into muscles usually occur in patients with severe disease. These symptoms

★ **TABLE 34-7** Clinical Findings in Deficiencies of Factors VIII and IX

Factor VIII or IX Level, Units/dL	Severity	Symptoms
<1	Severe	Frequent spontaneous hemarthrosis with crippling
		Frequent severe, spontaneous hemorrhage (intracranial, intramuscular)
1–5	Moderate	Bleeding at circumcision
		Infrequent spontaneous joint and tissue bleeds
		Excessive bleeding after surgery or trauma
		Serious bleeding from minor injuries
6–30	Mild	Rare spontaneous bleeds
		Excessive bleeding after surgery or trauma
		Might not be discovered until bleeding episode occurs

are not commonly seen in individuals with moderate or mildly severe disease. More characteristic of moderate hemophilia is excessive bleeding after traumatic injury. Mild deficiencies of FVIII or FIX can be asymptomatic and unsuspected until a surgical procedure or major traumatic injury results in severe bleeding. Although the site of bleeding varies from individual to individual, the clinical severity of deficiencies of both factors remains similar within families.

Laboratory Evaluation of the Hemophilias

Laboratory tests are required to screen for abnormalities of coagulation factors and then to confirm and quantitate the specific factor that is deficient (Chapter 36). Screening tests (APTT) are expressed as time, usually in seconds. Confirmatory assays are expressed in units of activity with normal plasma considered to have 1 unit (U) activity/mL, or 100 U/dL. The reference interval for both FVIII and FIX is ~50–150% of normal (0.5–1.5 U/mL).

Table 34-2 includes the results of screening tests in a variety of hemostatic disorders. The APTT is prolonged in both FVIII and FIX deficiencies; it is lengthened inversely to the level of factor present in the patient's plasma when the level is below the sensitivity of the testing methodology. Levels of ≤20 U/dL of FIX and ≤30 U/dL of FVIII consistently prolong the APTT (Chapter 36 and its Companion Resources).

Definitive diagnosis is made on the basis of the results of specific factor assays. Precautions should be used in interpreting results. (1) FVIII levels are lower in persons with blood group O than other blood groups (corresponding to the level of VWF) so the blood type must be considered when diagnosing FVIII deficiency. (2) FVIII level varies as an acute phase reactant and increases with exercise, inflammation, and so on. Estrogen-containing contraceptives also increase FVIII levels. (3) Screening test systems might not be sensitive enough to detect mild deficiencies at levels between 20 and 50 U/dL. In these cases, the physician may order a factor assay on the basis of the patient's history. (4) The newborn range for FIX is lower than that the adult range of 20–50% of normal, so one must refer to age-appropriate reference intervals when making the diagnosis of hemophilia B.

The results of additional laboratory tests shown in Table 34-3 are compared with the results in type 1 VWD. All platelet testing results are normal in the hemophilias. The thrombin time and PT are normal because neither assay depends on FVIII or FIX. One abnormal molecular variant, FIXBm, does cause prolongation of the PT when bovine brain thromboplastin is used instead of rabbit thromboplastin in the test system.[26] Tests for fibrinolysis also are normal.

Differential Diagnosis of Hemophilias

Hemophilia A must be distinguished from deficiencies of FIX or FXI and from VWD types 2N and 3. Hemophilia A is distinguished from hemophilia B by factor assays and from FXI deficiency by both factor assays and inheritance pattern. Prior to performing the factor assay, mixing studies in which the patient's plasma is mixed with normal plasma (1:1 ratio) with subsequent APTT testing should also be performed to eliminate the possibility of an inhibitor rather than the genetic disorder. In the event of an inhibitor, the APTT remains prolonged whereas with a factor deficiency, mixing corrects the defect.

Type 2N VWD is caused by abnormalities of the D′ domain of VWF that prevent FVIII from binding.[3] Both hemophilia and type 2N VWD demonstrate low FVIII levels and normal structure and functional tests for VWF:Ag, ristocetin cofactor activity, and VWF multimeric structure. Molecular testing may be required to distinguish hemophilia A from VWD type 2N, and patients with type 2N VWD would not respond clinically to FVIII replacement therapy. Patients with type 2N VWD exhibit an autosomal recessive inheritance pattern. Type 3 VWD, which also is an autosomal recessive disease, can be differentiated from hemophilia on the basis of an autosomal inheritance pattern and by a decrease of VWF:Ag in both platelets and plasma.

☑ CHECKPOINT 34-4

Referring to Table 34-3, explain why the platelet function tests are abnormal in VWD but not in FVIII or FIX deficiencies.

Carrier Detection and Prenatal Diagnosis

Daughters of hemophilic males are obligate carriers of the disease and generally do not require further testing. Daughters of obligate carrier mothers can inherit either one of their mother's X chromosomes and thus can be either carriers or normal. Hemophiliac males can inherit the disorder from carrier mothers or represent a spontaneous mutation. Female carriers of X-linked disorders are usually asymptomatic because they have one functional allele. However, recently it has been reported that up to a third of heterozygous females manifest mild hemophilia because of skewed X chromosome inactivation.[27]

Inactivation of one of the X chromosomes occurs randomly in each somatic cell of a female. Theoretically, in a carrier of an X-linked disorder, random inactivation results in approximately 50% of the cells having a functional X chromosome active, while the remaining 50% would have the X chromosome bearing the mutant allele. A female carrier of FVIII or FIX deficiency is expected to have approximately 50% of the normal plasma level of the factor in question. Detection of the carrier state cannot, however, be based merely on finding half of the normal activity in a factor assay because the normal reference interval is 50–150%. However, inactivation of the X chromosomes is not always randomly distributed. A carrier can have functional FVIII- or FIX-bearing X chromosomes in >50% of her hepatocytes, in which case her activity level would fall within the reference interval. Conversely, if >50% of the hepatocytes have the normal X chromosomes inactivated, she could show clinical signs of mild hemophilia. Also, the FVIII protein is an acute phase reactant and is physiologically increased in pregnancy, exercise, fever, and several other conditions, which can result in a transient rise of FVIII to within the reference interval. Both FVIII and FIX coagulant activity can rise with the use of oral contraceptives.

Detection of carriers is sometimes possible by analysis for both VWF antigen and FVIII activity levels. VWF:Ag levels in carriers should be ~2 times the FVIII:C.[28] The preferred method of detection of carriers is genetic testing when it is possible to establish the genetic mutation. Potential carriers of FIX deficiency can be detected by direct gene sequencing.[29,30] In families with a severe phenotype of hemophilia A, DNA testing using the Southern blot technique is available for screening for the inversion mutation in intron 22, the most commonly encountered mutation in severe deficiencies.

Prenatal diagnosis by genotypic analysis has certain advantages over phenotypic analysis. Results are not affected by X chromosome inactivation, ABO blood type, or VWF levels, and testing can be done earlier in gestation. Various methods are available for prenatal diagnosis; the method of choice varies with the type of mutation anticipated. A chorionic villus biopsy can be done at 11 weeks of gestation and tested with DNA studies such as restriction enzymes (restriction fragment length polymorphism [RFLP]) or PCR methods. Because of the enormous variety of different molecular defects and the considerable size of the FVIII gene, direct DNA diagnosis (PCR) is not available for all families. Direct DNA analysis is also limited by the fact that as many as one-third of hemophilia cases arise from new mutations. If the precise genetic defect is not known, it is difficult to do direct molecular analysis, but indirect DNA analysis (RFLP) may be informative.

Direct sampling of fetal blood from the umbilical vein is possible at many institutions, and a factor assay can be performed on the blood sample. Some mutations require the simultaneous analysis of both antigen and activity levels for identification. Ultrasound analysis for determining the gender of the fetus is also available. Patients often elect to undergo prenatal diagnosis (even when pregnancy termination is not being considered) so that physicians can take precautions at birth to prevent bleeding if the fetus is at risk for hemophilia.[31] Intracranial hemorrhage during vaginal delivery is a potentially life-threatening complication.

Therapy for Hemophilia

In general, the goal of treatment for bleeding in patients with a clotting factor deficiency as in hemophilia is increasing the clotting factor to a level sufficient to achieve hemostasis. Typically, hemostasis for minor bleeding can be achieved at plasma factor levels of 25–30% of normal, whereas severe bleeding requires at least 50% of normal activity. Patients with severe trauma or surgery require plasma levels of 75–100% of normal. For patients who are actively bleeding, several preparations are available to raise the level of FVIII or FIX in the patient's plasma (Table 34-8 ★).

Factor Replacement Therapy. FVIII deficiencies were originally treated with cryoprecipitate preparations or FVIII concentrate. FVIII concentrates are prepared from plasma by lyophilization, a freeze-drying process that results in a slight reduction of the activity of FVIII, and loss of the largest VWF multimers. In the past, major problems were encountered with the use of plasma-derived concentrates of FVIII, because the plasma from up to 20,000 donors is pooled to prepare one lot of product. Most patients who received this therapy before 1984 were exposed to hepatitis B, hepatitis C, and the HIV viruses. Since 1984, heat or solvent-detergent treatments have been used to inactivate the viruses, and concentrates are now considered safe. However, for a time, 90% of patients with severe FVIII deficiency had HIV antibodies and antibodies to hepatitis B surface antigen (indicating exposure to both viruses). Since 1987, no new cases of HIV have been attributed to the administration of clotting factor concentrates in North America, and transmission of hepatitis has been documented only rarely.

To prevent virus transmission, FVIII products can be prepared using monoclonal antibodies or recombinant technologies. Recombinant FVIII (rFVIII), however, also has hazards. Human albumin was added to the "first generation" rFVIII preparations to stabilize the protein, and this has been associated with transmission of the B19 parvovirus (although there have been no significant clinical sequelae from these transmissions). Full-length FVIII products stabilized by sucrose, not human albumin, are also available. Another form of FVIII replacement therapy is a rFVIII in which the B domain is deleted. This type of rFVIII does not require albumin additive for stabilization. Concentrates can be used by hemophiliacs at home as prophylactic therapy to prevent extensive bleeding; they have markedly reduced the crippling hemarthropathy and improved the quality of life for patients with severe disease.

An alternative form of therapy in patients capable of producing some FVIII (mildly affected hemophilia A) is DDAVP, which stimulates endothelial cells to release FVIII and VWF into the plasma (see discussion under VWD therapy).

FIX deficiency can be treated with whole plasma or with concentrates that also contain factors II, VII, and X (prothrombin complex concentrates [PCC]). Another complication of therapy with intermediate purity FIX products (PCC) in addition to viral infections is thrombosis because these concentrates contain variable amounts of activated factors VII, X, and prothrombin. Purified FIX concentrate (Mononine®) is prepared from plasma using monoclonal antibodies. Purified FIX concentrates are heat treated to inactivate the hepatitis and HIV viruses as described for FVIII. Recombinant FIX (Benefix®) is also available and is now the preferred form of therapy, avoiding complications of both viral transmission and hypercoagulability.

Gene Therapy for Hemophilia. The potential use of gene therapy to achieve a cure for both FVIII and FIX deficiencies is being actively investigated.[32,33] Because of the larger size of the FVIII molecule (~200 kDa) compared with the FIX protein (44 kDa), it has been more difficult to successfully express FVIII using gene transfer protocols. The first clinical trials in FIX deficiency were promising, but limited benefits and complications resulted in the trials' termination prematurely.[34] Progress in the development of gene therapy for the hemophilias has been slow. Initially the increases in clotting factor activity were transient, and problems were associated with the viral vectors used to transfer the DNA in the early studies. Subsequent clinical trials demonstrated long-term expressions of therapeutic FIX levels; however, the levels were not sufficient to correct bleeding from trauma.[32] Currently the cornerstone of treatment of hemophilias remains replacement by recombinant products. However, most

★ **TABLE 34-8** Therapy for Hemophilia

Modes of therapy for FVIII deficiency
- Recombinant FVIII (rFVIII)
- FVIII concentrates
- Cryoprecipitate
- Deamino-8-D-vasopressin (DDAVP) (for some)
- Gene therapy (in research phase)

Modes of therapy for FIX deficiency
- Recombinant FIX (Benefix)
- Prothrombin complex concentrates (e.g., Konyne, Proplex)
- FIX concentrates (e.g., Mononine)
- Gene therapy (in research phase)

researchers in the field believe that the likelihood of eventual success in gene therapy of hemophilia is high.[27] Recently, an Anglo-American group reported on six patients with severe hemophilia B who had been treated using an AAV vector, and all converted to a moderate or mild hemophilia phenotype. Clinical trials reopened in March 2012 and participants have been able to reduce or eliminate the need for regular FIX infusions.[35]

Inhibitors. Some hemophilia patients form neutralizing antibodies, also called *inhibitors*, to their deficient factor after exposure to factor-replacement therapies. In vivo antibody formation causes destruction of the infused factor, neutralizes the coagulant effects of therapy, and complicates treatment of the patient. An inhibitor to FVIII or FIX is clinically suspected when a bleeding episode fails to respond to an adequate dose of factor concentrate. Approximately 5–20% of hemophilia A patients and 1–3% of hemophilia B patients have inhibitors. There is no way to accurately predict which patients will form inhibitors. The prevalence of antibody formation is higher in patients with severe disease than those with moderate or mild disease. FVIII inhibitors are twice as common in African Americans than in Caucasians, while Scandinavians are at higher risk than other populations to develop FIX inhibitors. Gene lesions resulting in CRM− or severely reduced phenotypes are associated with a higher risk of developing inhibitors compared with CRM+ phenotypes. Patients with large FVIII gene deletions (affecting >1 domain of the FVIII molecule) have a threefold higher risk of developing an inhibitor compared with single domain deletions.[36]

CASE STUDY (continued from page 697)

Scott's mother was questioned regarding the family history. She stated that her father and his brother had had similar bleeding symptoms. Her father died from a brain hemorrhage, and his brother died from complications associated with HIV. These brothers had sisters, none of whom had bleeding problems.

3. What type of inheritance is most probably present in this family?
4. Is this history typical of that of a patient with von Willebrand disease? Why?
5. What could have caused the patient's great uncle to have acquired HIV infection?

Autosomal Recessive Disorders

Autosomal recessive traits are expressed only in those individuals homozygous (or double heterozygous) for the defective gene who inherit an abnormal allele from each parent. Each parent is likely to be heterozygous for the trait. Individuals who are homozygous generally have bleeding symptoms; those who are heterozygous usually have normal hemostasis.

Hereditary deficiencies of the remainder of the coagulation factors are rare in most of the world's populations. In areas of the world where consanguinity (mating between relatives) is more common, the prevalence of autosomal recessive inherited factor deficiencies is higher and can approach that of hemophilia B.[37]

The genetic mutations for all the proteins to be discussed are diverse between families but unique and constant within each family group. The mutation type and site within the molecule determines the severity of bleeding symptoms. Some mutations result in CRM+, and others result in CRM− or CRM^R phenotypes.

The clinical expression of the autosomal inherited deficiencies varies. Deficiencies of some factors result in severe bleeding symptoms while others are not associated with any bleeding abnormalities. Bleeding phenotype even among individuals with the same disorder can vary significantly. Deficiencies of some fibrinolytic inhibitors can also result in bleeding symptoms. The conditions are discussed under the appropriate categories.

Coagulation Factor Disorders with Bleeding Symptoms

The diagnosis of the bleeding disorder is suspected from the results of the PT and APTT and confirmed with subsequent specific factor assays (Table 34-2). The degree of abnormality suggested by both PT and APTT can be small in cases of mild deficiency. Tests for platelet number and function are normal as are tests for fibrin degradation products and the thrombin time with some exceptions. Before a hereditary disease is considered, however, all possible causes for acquired coagulation factor deficiencies should be ruled out.

The autosomal recessive bleeding disorders are discussed in numerical order beginning with fibrinogen (factor I) deficiencies. Rare hereditary deficiencies of hemostatic proteins other than those involved in fibrin formation are presented briefly. Additional information including clinical bleeding characteristics and therapy can be found on this text's Companion Resources. Table 34-9 ★ shows the results of laboratory tests in these disorders.

Fibrinogen (Factor I) Deficiency. Congenital fibrinogen disorders can be classified as quantitative (type I, afibrinogenemia or hypofibrinogenemia) or qualitative (type II, dysfibrinogenemia). Afibrinogenemia accounts for ~23% of inherited abnormalities, hypofibrinogenemia for ~26%, and dysfibrinogenemia for 51%.[38]

Two forms of fibrinogen deficiency are inherited as autosomal recessive traits. **Afibrinogenemia** is a homozygous form of the disease in which no chemically, antigenically, or functionally detectable fibrinogen is found. **Hypofibrinogenemia** is a heterozygous form in which plasma levels of fibrinogen are ~50% of normal (reference interval: 200–400 mg/dL). Consanguinity is found in about half of the families with afibrinogenemia. The prevalence of afibrinogenemia is estimated to be 1 in 1,000,000. More than 80 novel mutations have been identified in patients with afibrinogenemia, and often the same mutations in the heterozygous state have been identified in hypofibrinogenemia.[38] A database of mutations is available at www.geht.org/databaseang/fibrinogen/.

Clinically, afibrinogenemia is the more severe disease with patients having a severe bleeding disorder. At birth, umbilical cord and mucosal bleeding are frequent symptoms and can lead to death, but in general, patients have a milder course than do severe hemophiliacs and can go long periods without bleeding episodes. Joint or uterine bleeding is seen in 50% of patients.[37] Fatal bleeds from intracranial hemorrhages in infants have been reported. Patients with

★ **TABLE 34-9** Laboratory Screening Tests in Autosomal Recessive Coagulation Factor Disorders

	Platelet Tests		Coagulation Factor Tests					
Factor	**Count**	**CT**	**PT**	**APTT**	**TT**	**FibA**	**Tests for FDP**	**Other**
Afibrinogenemia	±N	±A	A	A	A	Absent	N	Abnormal platelet aggregation with ADP and epinephrine
Hypofibrinogenemia	N	N	N	N	A	N	N	
Dysfibrinogenemia	N	N	N	N	A	Variable	N	
Factor II	N	N	A	A	N	N	N	
Factor V	N	±N	A	A	N	N	N	
Factor VII	N	N	A	N	N	N	N	
Factor X	N	N	A	A	N	N	N	Abnormal Russell's viper venom test
Factor XI	N	N	N	A	N	N	N	
Factor XII	N	N	N	A	N	N	N	No bleeding tendency
Prekallikrein	N	N	N	A	N	N	N	No bleeding tendency; APTT corrected with 10-minute incubation with kaolin reagents
HK	N	N	N	A	N	N	N	No bleeding tendency
Factor XIII	N	N	N	N	N	N	N	

CT = closure time; PT = prothrombin time; APTT = activated partial thromboplastin time; TT = thrombin time; FibA = fibrinogen assay; FDP = fibrin degradation products; N = normal; A = abnormal; HK = high molecular weight kininogen

hypofibrinogenemia have a milder bleeding course. They are often asymptomatic, but bleeding can follow invasive procedures. Both disorders are associated with recurrent pregnancy loss as well as antepartum and postpartum hemorrhage.[39]

In afibrinogenemia, all laboratory tests based on production of a fibrin clot (PT, APTT, thrombin time) are abnormal, and all are corrected in mixing studies with normal plasma. The bleeding time is prolonged in about half of the patients because fibrinogen is required for primary platelet aggregation. Platelet aggregation tests are also abnormal (Table 34-9). The diagnosis is confirmed using antigenic and functional assays for fibrinogen, which usually reveal <1 mg/dL of the protein. The platelet count is also decreased in ~20% of patients. The erythrocyte sedimentation rate approaches zero because of the lack of fibrinogen in the plasma.

The presence of heparin, fibrin degradation products, or circulating anticoagulants in the plasma can also prolong the hemostasis screening tests, and must be considered in the differential diagnosis of afibrinogenemia. Mixing studies, the patient's history, and tests for FDPs are helpful in distinguishing inherited afibrinogenemia from acquired conditions in which fibrinogen can be decreased (Chapters 32 and 36).

Replacement therapy with cryoprecipitate or fibrinogen concentrates is used when patients are actively bleeding or to prevent excessive hemorrhage during surgical procedures.

In the third form of fibrinogen abnormality, **dysfibrinogenemia**, the patient has normal levels (mg/dL) of fibrinogen but has abnormal fibrinogen molecules in the plasma. Dysfibrinogenemia is a relatively rare disorder that most commonly occurs in a heterozygous state. More than 600 novel mutations have been associated with dysfibrinogenemia.[38] Mutations have been identified in all three genes encoding the fibrinogen peptide chains (*FGA*, *FGB*, or *FGG*).[39,40] Similar to abnormal hemoglobins, the fibrinogen abnormalities are often named for the city in which they were discovered. Some individuals have both reduced antigen levels and variant fibrinogen molecules and constitute a subcategory called *hypodysfibrinogenemias*.[38]

Dysfibrinogenemia is inherited as an autosomal dominant trait. Clinically, ~50% of individuals with dysfibrinogenemia have no bleeding symptoms or other clinical manifestations and are discovered incidentally when laboratory tests are ordered for unrelated reasons and unexpected abnormal results are found. Approximately 25% of individuals have bleeding complications, and 25% have thrombosis (Chapter 35). It is suspected that the number of identified patients with dysfibrinogenemia is a small percentage of the actual number present in the population. In those patients who do exhibit hemorrhagic symptoms, the bleeding is mild and generally occurs only after trauma. Dysfibrinogenemia has also been associated with hereditary renal amyloidosis.[38,41] Clinical manifestations depend on the type and the location of the mutation in the protein. Differential diagnosis includes acquired dysfibrinogenemia, which can result from liver disease or, rarely, pancreatitis, paraneoplastic syndrome, or renal carcinoma.[41]

The functions or properties of fibrinogen are also affected by the type and site of mutation. In general, these mutations impair either the conversion of fibrinogen to fibrin monomer (proteolysis step), the conversion of fibrin monomers to polymers (spontaneous polymerization step), or the cross-linking of the fibrin polymers. Some mutations affect either thrombin binding or fibrinopeptide cleavage and are associated with abnormal release of either fibrinopeptides A or B. Other mutations affect the polymerization sites within the N-terminus of the α- or β- chain or the C-terminal region of the γ- or β- chains (Chapter 32).

Laboratory tests for hemostasis in patients with dysfibrinogenemia are usually normal with the exception of the thrombin time, clot-based quantitative fibrinogen assays, and reptilase time, which are prolonged in most patients. However, determinations of fibrinogen antigenically or by a biochemical technique of precipitation and quantitation of fibrinogen are normal. Bleeding times and other platelet tests also are normal.

☑ CHECKPOINT 34-5

Explain why the thrombin time is abnormal in patients with afibrinogenemia and dysfibrinogenemia.

Prothrombin (Factor II) Deficiency. Deficiencies of prothrombin occur at an estimated prevalence of 1 in 2,000,000, making it one of the rarest inherited bleeding disorders. Prothrombin deficiency is genetically heterogeneous with >50 mutations identified, including both quantitative (type I, hypoprothrombinemia) and qualitative (type 2, dysprothrombinemia) deficiencies (www.hgmd.cf.ac.uk/ac/gene.php?gene=F2). Combined defects have also been reported. Heterozygotes have prothrombin levels of ~50% of normal and are usually asymptomatic; homozygotes with activity levels <10% of normal experience severe bleeding manifestations; complete absence of prothrombin appears to be incompatible with life.[42] Treatment, if needed, is prothrombin complex concentrates (PCC). Both the PT and APTT are typically prolonged while the thrombin time and bleeding time are normal (Tables 34-2 and 34-9). The degree to which the PT and APTT are prolonged varies from patient to patient, and results can occasionally be within the reference interval. The diagnosis is established using a specific factor assay for functional prothrombin and immunologic tests for antigen levels.

Factor V Deficiency. The first reported case of FV deficiency was presented by Owren in 1947.[43] The prevalence of FV deficiency is 1:1,000,000, and both quantitative (type 1) and qualitative (type 2) disorders have been described (www.hgmd.cf.ac.uk/ac/gene.php?gene=F5). So far, 112 specific genetic defects have been identified. The only replacement therapy available, if needed, is fresh frozen plasma. Like prothrombin and FX, FV functions in the common pathway, so the PT and APTT are both prolonged, but thrombin time (TT) is normal (Table 34-2). Abnormal bleeding times are reported in about one-third of patients and may be related to a deficiency of FV in platelet α-granules.[37] Other screening tests are normal (Table 34-9). Definitive diagnosis requires a specific FV assay (functional and immunologic).

Factor VII Deficiency. FVII deficiency is the only plasma coagulation factor deficiency in which the PT alone is prolonged (Tables 34-2 and 34-9). The incidence is estimated as 1 in 500,000, making it the most common of the rare inherited coagulation disorders.[42] To date, nearly 270 cases have been reported, and both quantitative (type 1) and qualitative (type 2) disorders have been described (www.hgmd.cf.ac.uk/ac/gene.php?gene=F7). A quantitative FVII determination by standard factor assay methods (functional and immunologic) provides a definitive diagnosis. Homozygous patients usually have <10 U/dL of the factor; heterozygous individuals (who are generally asymptomatic) have 40–60 U/dL. It is important to use age- and gestational-related reference intervals because FVII is naturally low at birth.[39] Therapeutic options include recombinant FVIIa (optimal replacement therapy), PCC, and plasma-derived FVII concentrates.[42]

☑ CHECKPOINT 34-6

Explain why the prothrombin time but not the APTT is prolonged in FVII deficiency.

Factor X Deficiency. The incidence of FX deficiency is 1 in 1,000,000 in the general population.[42] FX deficiency is genetically heterogeneous, and ~100 distinct mutations, both quantitative (type 1) and qualitative (type 2), have been described (www.hgmd.cf.ac.uk/ac/gene.php?gene=F10).[44,45] Both the PT and APTT are usually prolonged (Tables 34-2 and 34-9). However, in three mutations, the PT is prolonged, but the APTT is normal; the opposite is true in another variant.[46] The Russell's viper venom (RVV) test, which directly activates FX, is prolonged, although it too can be normal in some variants. A FX assay (functional and immunologic) is the definitive test although it is important to exclude vitamin K deficiency before confirming the diagnosis. Treatment options for symptomatic patients are limited to fresh frozen plasma (FFP) and PCC.

Factor XI Deficiency. FXI deficiency, sometimes referred to as hemophilia C, is the fourth most common inherited bleeding disorder[37] with an estimated frequency in the general population of 1 in 100,000.[42] FXI deficiency has a high frequency in the Ashkenazi Jewish population; approximately 0.2% of these individuals are homozygous and ~8% are heterozygous for this disorder.

Laboratory screening tests reveal a prolonged APTT and normal PT although the APTT can be normal in heterozygous patients with mild deficiency. Other tests are normal (Table 34-9). Deficiencies of factors XII, XI, VIII, and IX, prekallikrein (PK) and high molecular weight kininogen (HK) are considered when the APTT is the sole abnormal screening test. The clinical and family histories are useful in determining which factor assay to perform. The specific assay for FXI is the definitive test for this deficiency. Homozygous individuals have from <1 U/dL up to 10 U/dL FXI activity. Most patients have equivalent decreases in antigenic and functional activity, indicating a type I (quantitative) disorder. A few (~7) FXI mutations are associated with production of a dysfunctional protein.[42,47] More than 180 mutations in the FXI gene have been associated with FXI deficiency, although two different point mutations account for >90% of cases of FXI deficiency in Ashkenazi Jews (www.hgmd.cf.ac.uk/ac/gene.php?gene=F11).[47] Factor XI concentrates are available in Europe, but treatment options for symptomatic patients in the United States are limited to FFP and low dose rFVIIa.

Laboratory testing for FXI activity requires precautions in collecting and handling the specimen. If the blood sample is collected in glass tubes, FXI can become activated (glass pre-activation of contact proteins; Chapter 32) and can lead to false normal results and missed diagnosis of mild deficiencies. It is recommended that blood be drawn in plastic to minimize glass contact activation. Multiple freezing and thawing of the specimen and/or a delay in running the assay (prolonged plasma storage) have been reported to cause preactivation of FXI and can result in normalization of an abnormal APTT. However, a single quick freeze and quick thaw appears to have minimal effect on FXI levels (Chapter 36 and the Chapter 36 Companion Resources). The type of activator used by different manufacturers can also affect a test system's ability to detect a deficiency. Patients with mild FXI deficiencies may react variably with different activators. Abnormal results can be obtained with one reagent and normal results with another.

Factor XIII Deficiency. FXIII deficiency is rare with an estimated prevalence of 1 in 2,000,000. FXIII deficiency is a highly heterogeneous disorder, and a wide variety of genetic mutations affecting either the A- or B-chain of FXIII have been reported. FXIII deficiency is now classified as FXIII-A deficiency, FXIII-B deficiency, and a possible combined FXIII-A and FXIII-B deficiency. To date, >100 mutations of the *F13A1* gene have been identified (www.hgmd.cf.ac.uk/ac/gene.php?gene=F13A1), whereas only 6 mutations affecting the *F13B* gene have been identified (www.hgmd.cf.ac.uk/ac/gene.php?gene=F13B). Patients generally lack both plasma and platelet FXIII.[48] Platelet FXIII contains only the A2 form of FXIII (Chapter 32), which carries the active enzymatic site. However, inherited deficiencies of the B-chain also result in low plasma FXIII levels because the B-chains are required for the stabilization and survival of FXIII (A2B2) in plasma. Low plasma levels of FXIII (~5% of normal) are generally sufficient to control bleeding. The hallmark of FXIII deficiency is bleeding from the umbilical cord site, after circumcision, or spontaneous intracranial bleeding after delivery. Miscarriage is very common because stabilization of fibrin at the maternal–fetal interface is required to maintain pregnancy.[48] In 2011, the U.S. Food and Drug Administration (FDA) approved a FXIII concentrate for treatment of inherited FXIII deficiency.[49]

Because FXIII functions after formation of fibrin, a deficiency does not affect the usual screening tests (PT, APTT, TT). However, in vitro clot formation is abnormal. Excess red cells are present at the bottom of a whole blood clot tube after clot retraction. Laboratory diagnosis has relied on a screening test for FXIII based on dissolution of the fibrin clot in 1% monochloroacetic acid or 5M urea. This *clot solubility test* is positive (i.e., the clot dissolves) when the FXIII concentration is 0.5 U/dL or less. The clot is insoluble at levels as low as 1–2 U/dL (Chapter 36). It has been reported that this test can detect only severe FXIII deficiency, has poor reproducibility and sensitivity, and is now not recommended as a screening test for this deficiency.[50] Because it is not sensitive to mild deficiencies that can result in clinical sequelae, efforts have been made to develop more sensitive tests.[51] Specific assays are available, some that measure enzymatic activity and others that use immunologic techniques such as ELISA. The reference interval for F-XIII is about 14–28 mg/L.[51]

Combined Factor V and Factor VIII Deficiency (F5F8D). In this rare disorder (<100 families identified), both FV and FVIII levels are reduced (5–30% of normal levels).[42,52] The antigen and clotting activity are usually concordant. This disorder is also called *familial multiple clotting factor deficiency type 1*. Bleeding is mild to moderate and similar to that observed in other coagulation disorders. Combined FV/VIII deficiency is caused by a mutation of either of two genes.[52] The first gene produces a protein of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC 53) called L-mannose 1 (LMAN1), and mutations of this gene (*LMAN1*) are found in ~70% of patients with this disorder (~34 identified; www.hgmd.cf.ac.uk/ac/gene.php?gene=LMAN1). The second is the multiple coagulation factor deficiency 2 gene (*MCFD2*), which accounts for the remaining 30% of patients (17 mutations identified; www.hgmd.cf.ac.uk/ac/gene.php?gene=MCFD2). The LMAN1-MCFD2 proteins form a complex, which functions to facilitate transport of FV and FVIII from the endoplasmic reticulum to the Golgi apparatus. The decrease in FV and FVIII results from defective intracellular transport and secretion in a pathway unique to these two coagulation factors.[52] The PT and APTT are prolonged in this disorder. If therapeutic factor replacement is required, both FFP (to replace FV) and a FVIII-containing product or DDAVP can be used.

Combined Deficiencies of Vitamin K-Dependent Clotting Factors. *Vitamin K clotting factor deficiency (VKCFD)* is caused by mutations of either the gene for γ-glutamyl carboxylase, the enzyme responsible for the γ-carboxylation reaction (*GGCX*; VKCFD1), or the gene for vitamin K epoxide reductase (*VKORC1*; VKCFD2).[42,52] VKCFD is characterized by deficient activity of all vitamin K-dependent clotting factors. γ-carboxylation is required for the production of functional forms of these clotting factors and enables them to interact with phospholipids in the formation of the prothrombinase and Xase complexes (Chapter 32). In this disorder, the proteins are produced but are nonfunctional because they lack γ-carboxylation. Affected individuals resemble patients on Coumadin therapy with drastically prolonged PT and APTT tests and normal TT.

☑ CHECKPOINT 34-7

Explain why the laboratory screening tests are normal in patients with FXIII deficiency.

Factor Deficiencies without Clinical Bleeding

The combination of a normal PT, a prolonged APTT, and a negative history of bleeding suggests deficiencies of FXII, PK (prekallikrein), or HK (high-molecular-weight kininogen). No bleeding symptoms are associated with deficiencies of these proteins even after severe trauma or during surgery. When an abnormal APTT is found in presurgical or screening testing, it should be resolved.[53] Although deficiencies of FXII, PK, or HK display abnormal plasma coagulation in vitro, none is associated with in vivo bleeding. However, these deficiencies must be distinguished from those of FVIII, FIX, and FXI, which are accompanied by defective hemostasis. The abnormal APTT is corrected by 1:1 mixing studies with normal plasma. All other hemostatic screening tests are normal (Table 34-9). The three deficiencies are differentiated by specific factor assays performed by the traditional modification of the APTT or by chromogenic substrate assays (Chapter 36).

The specific factor assay can be preceded by a laboratory screening procedure (a modified APTT) to presumptively identify whether PK is the deficient factor and to choose assay procedures to perform. If the patient's plasma is incubated with a kaolin- or celite-containing APTT reagent for 10 minutes (rather than the usual 2 or 3 minutes), the prolonged APTT is corrected to normal in a PK deficiency.[53] The APTT remains prolonged in FXII or HK deficiencies.

A deficiency of HK (also called *Fitzgerald*, *Williams*, or *Flaujac factor*) is one of a group of disorders involving not only HK but also variable deficiencies of low-molecular-weight kininogen (LK). LK does not affect the hemostatic system, so an isolated LK deficiency would not affect laboratory coagulation tests. Concurrent absence of the LK aids in classifying the disorders, however. "Williams" trait was characterized by a deficiency of both HK and LK; "Fitzgerald" and "Flaujac" traits were deficient in only HK.[54] Because there is no clinical bleeding, therapy is usually not needed for these conditions.

Disorders of Fibrinolytic Protein Inhibitors with Bleeding Symptoms

Congenital deficiencies of these disorders are rare.[55] Deficiencies of α_2-antiplasmin (AP) and plasminogen activator inhibitor-1 (PAI-1) result in impaired regulation of fibrinolysis with excess plasmin activity. This disturbs the balance between coagulation and fibrinolysis, resulting in a bleeding disorder because of hyperfibrinolysis. Initial hemostasis is typically normal, but delayed bleeding can occur because of premature lysis of hemostatic plugs. Routine screening coagulation tests are normal. Specific assays for PAI-1 or AP would reveal variably reduced levels. Further information is available on this text's Companion Resources.

CASE STUDY *(continued from page 702)*

Screening hemostasis tests were performed on Scott's blood in the hemostasis laboratory. The results follow (see reference intervals in table D on cover):

Platelet count	250 × 10^9/L
Prothrombin time	12 sec
Activated partial thromboplastin time	95 sec

6. Name the coagulation factor deficiencies that are possible with these laboratory results.
7. What is the most likely factor deficiency? Why?

Acquired Disorders of Hemostasis Associated with Bleeding

Acquired deficiencies of hemostatic proteins that result in bleeding symptoms can occur in individuals who were previously normal. Acquired deficiencies are far more common than hereditary disorders. Acquired coagulation disorders are also more complicated than inherited conditions because multiple factors usually are deficient, and bleeding is often simultaneous from more than one site. In addition to acquired deficiencies of the fibrin-forming and fibrinolytic proteins, there are also deficiencies of naturally occurring inhibitors.

Acquired disorders occur in response to another disease process and are produced by a variety of mechanisms. The conditions in which these disturbances occur are classified into the following categories: disseminated intravascular coagulation, primary fibrinogenolysis, liver disease, vitamin K deficiency, and acquired pathologic inhibitors.

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is a condition in which the normal balance of hemostasis is altered, allowing the uncontrolled and inappropriate formation and lysis of fibrin within the blood vessels. Activation of coagulation occurs systemically rather than locally at sites of vascular injury. Fibrin is deposited diffusely within the capillaries as well as in arterioles and venules. As fibrin is formed, several clotting proteins and naturally occurring inhibitors and platelets are consumed faster than they are synthesized (**consumption coagulopathy**). The result is an acquired deficiency of multiple hemostatic components. Fibrinolysis follows fibrin formation as a natural sequence of the hemostatic process, the same processes that occur in normal hemostasis except that they happen at the wrong time and in the wrong place. As a result of consumption of coagulation factors and platelets and the formation of fibrin degradation products (FDP), the patient often bleeds at the same time that disseminated clotting is occurring.

Incidence

DIC occurs in approximately 1 in 1000 hospitalized patients. About 20% of the cases are asymptomatic and suspected only on the basis of laboratory data. It can occur at any age although it is more often seen in the very young and the elderly.

Etiology

DIC is a syndrome, not a disease. It is a group of symptoms that is always triggered by a primary condition that does not necessarily involve coagulation. A number of diverse disease states can trigger the DIC syndrome; they often involve the introduction of tissue factor (TF) into the vascular system, resulting in the initiation of fibrin formation. TF can enter the blood from mechanical injury to tissues or from injury to endothelial cells.

Conditions most often associated with triggering DIC are summarized in Table 34-10 ★. The most common cause is infections, particularly those associated with septicemia. The trigger mechanism of infections is likely bacterial toxins, cytokines (IL-1, IL-6, and/or

★ **TABLE 34-10** Clinical Conditions Associated with the Development of Disseminated Intravascular Coagulation (DIC)

Infections	Bacterial (endotoxins)
	Viral
	Fungal
	Rickettsial
	Protozoal
Complications of pregnancy	Abruptio placentae
	Amniotic fluid embolism
	Retained placenta
	Toxemia
	Intrauterine fetal death
	Septic abortion
Neoplasms (malignant)	Solid tumors
	Leukemia, particularly acute promyelocytic
Massive tissue injury	Burns
	Trauma
	Head injury
	Extensive surgery
	Extracorporeal circulation
Vascular injury	Shock
	Hypotension
	Hypoxia
	Acidosis
Miscellaneous	Snake bite
	Heat stroke
	Any disease

tumor necrosis factor) released into the tissues by the inflammatory response and activate endothelial cells. Resting endothelium does not express TF, but endothelial cells or monocytes activated by inflammatory cytokines or injured by endotoxin do express TF activity. The full DIC response is seen in 30–50% of patients with gram-negative or gram-positive septicemia.[56]

Complications of pregnancy likely cause DIC because amniotic fluid acts as a thromboplastin to activate fibrin formation pathways. With massive tissue or blood cell injury, TF and cytokines (similar to acute infections) are thought to enter the circulation and activate coagulation. In one study, most patients with head trauma had laboratory evidence of DIC caused by procoagulants entering the circulation from the injured brain tissue.[57] The trigger mechanism of malignant cells is a variation of tissue injury. Some malignant cells express TF, and others have been shown to produce a cysteine protease (cancer procoagulant) capable of directly activating FX.[56,58] Figure 34-4 summarizes the potential activation sites in the hemostatic system.

Pathophysiology

In response to the initiating event, thrombin is formed within the circulation. Unlike the physiologic formation of the hemostatic plug in which thrombin generation remains limited and localized at the site of vessel injury, DIC results in generalized or systemic activation of coagulation. The circulating thrombin acts on its substrates as they circulate in the same manner as it does after an injury-induced localized formation of fibrin (Chapter 32). This unregulated generation of thrombin results in the consumption of fibrinogen; factors V, VIII, and XIII (the natural substrates of thrombin); and depletion of prothrombin (the precursor zymogen). Thrombin is a potent agonist of platelets inducing platelet activation and aggregation. Thrombin also binds to receptors on endothelial cells, inducing endothelial release of tissue plasminogen activator (tPA), which in the presence of the newly formed fibrin activates plasminogen to plasmin and triggers an aggressive secondary fibrinolysis. As plasmin is generated, plasminogen becomes depleted. DIC results from a failure of

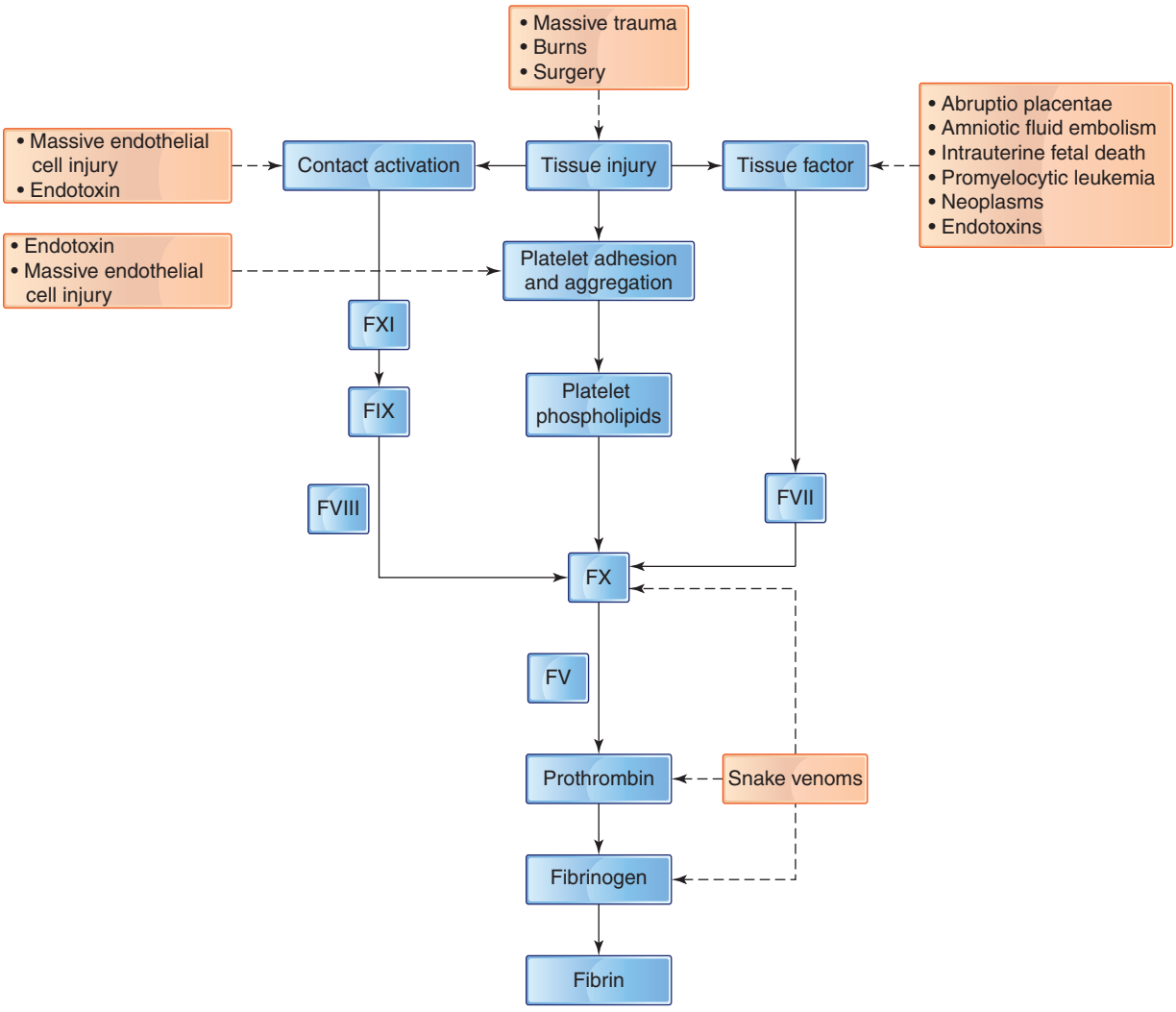


FIGURE 34-4 Conditions that initiate DIC activate the hemostatic pathways in a variety of ways. Solid arrows show normal hemostasis. Processes that trigger DIC are shown in the boxes, and broken arrows indicate proposed sites of activation.

the mechanisms that limit blood clotting and thrombin generation (the normal inhibitory pathways to prevent the systemic effects of thrombin).

The coagulation inhibitors antithrombin (AT), heparin cofactor II (HCII), and thrombomodulin (TM), normally effective in regulating the localized generation of thrombin, are overwhelmed in DIC. Deficiencies of AT, protein C (PC), and protein S (PS) are induced because they are utilized in removing their activated substrates from the circulation. Also, IL-1 and tumor necrosis factor (elevated in sepsis) decrease TM expression on endothelium, resulting in decreased activation of the PC/PS inhibitory system (Chapter 32).

Plasma levels of fibrinopeptides A and B (FPA and FPB) and D-dimer are elevated because of the actions of thrombin on fibrinogen and of plasmin on fibrin. FDPs interfere with fibrin formation and platelet function, contributing to the bleeding tendency.

All of these events can result in bleeding as the clotting mechanism is activated and procoagulant components become depleted. The pathogenesis of DIC is summarized in Figure 34-5 ■.

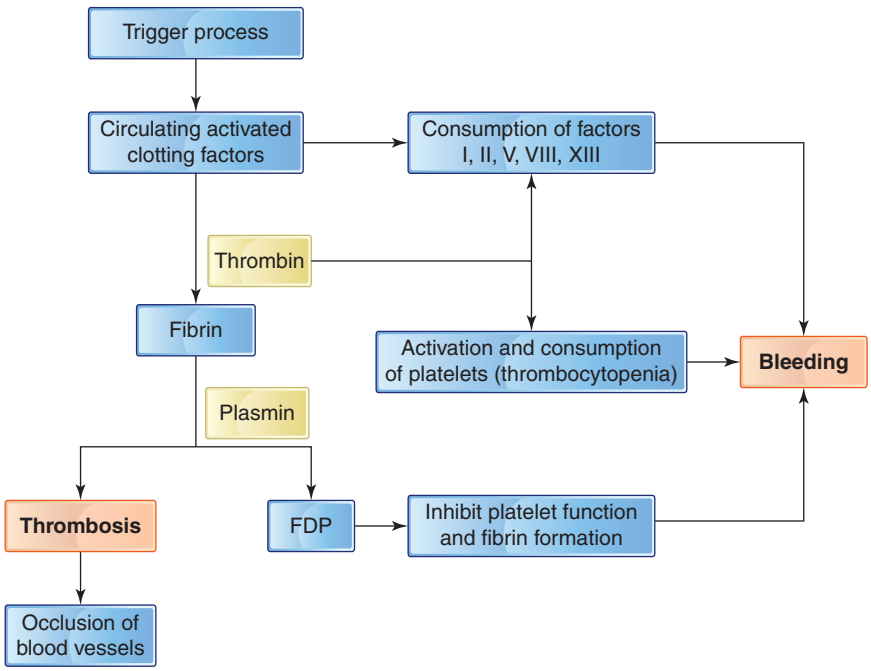
Clinical Aspects of DIC

The symptoms seen in patients with DIC result from the presence and activation of thrombin, plasmin, platelets, endothelium, and proteolytic inhibitors within the bloodstream and vary from patient to patient because of the complex interactions between these components. Because clotting factors and platelets are consumed, bleeding symptoms are favored in some patients. In other patients, thrombosis is the dominant process. In addition, the intensity and duration can result in either an acute or chronic clinical course. Patients with acute DIC tend to manifest hemorrhagic symptoms whereas in those with chronic DIC, thrombosis is more likely to predominate.[58] The more commonly recognized acute form begins with sudden onset of severe bleeding and is seen in 80–90% of patients with DIC. In the chronic form (10–20% of patients), the stimulus that triggers clotting is weaker, and the natural homeostatic mechanisms are sufficient to replace depleted hemostatic components.

Hemorrhages and thrombosis occur predominantly in the microvasculature and are responsible for the clinical manifestations. In patients with acute DIC whose disease course is hemorrhagic, bleeding begins abruptly and generally occurs from multiple sites simultaneously. Sites of bleeding tend to correspond to the tissues involved in the triggering event. Possible bleeding manifestations include hematuria; gastrointestinal and respiratory tract bleeding; intracranial bleeding; epistaxis; oozing from needle puncture sites, surgical drains, or sutures; and spontaneous bruising and petechiae. Bleeding can be profuse, leading to death.

At the same time, small strands of fibrin (microclots) form inside blood vessels and obstruct the microvasculature. Because blood vessels are occluded, tissue anoxia and microinfarcts in various organs can occur. Manifestations can include renal failure, coma, liver failure, respiratory failure, skin necrosis, gangrene, and venous thromboembolism.

Shock is a common feature and can be either a cause or an effect of DIC. The association of shock with DIC is complex and not well understood. Shock is likely induced because cytokine generation and products of the kinin and complement systems cause increased vascular permeability and hypotension. In spite of advanced improvement in clinical care, the mortality rate of DIC is still high, approximately 50–60%.



■ **FIGURE 34-5** Pathogenesis of DIC. Activation of hemostasis by triggering processes leads to clotting and bleeding simultaneously. Intravascular fibrin formation results in occlusion of vessels. During clotting, several coagulation factors and platelets are consumed, leading to bleeding.

Laboratory Evaluation of DIC

The physician suspects the diagnosis of DIC primarily based on the patient's clinical symptoms in the presence of an underlying disorder known to be associated with DIC. Laboratory tests are ordered to support a suspected clinical diagnosis. No single laboratory test will establish a diagnosis of DIC, nor is any combination of tests specific for DIC. Screening tests that are usually ordered by the physician include a platelet count, blood smear, PT, APTT, fibrinogen level, and D-dimer test. Some laboratories include a test for AT. These tests demonstrate the generation of both thrombin and plasmin and can reflect the severity of the consumption of hemostatic components. Refer to Table 34-11 ★ for typical laboratory results in patients with DIC.

The platelet count, the most useful parameter, can fall to levels of 40–75 $\times$ 10^9/L. It is decreased in 97% of patients with acute DIC. Identifying a decrease in the platelet count in patients whose usual platelet count is in the upper reference interval can be difficult, and serial platelet counts that clearly demonstrate decreasing values are more useful than a single determination.

The PT and APTT can be prolonged because of the decrease in factors II, V, and VIII and fibrinogen although their alterations are not as consistent as the platelet count. One or both of these tests are prolonged in 60–75% of patients with DIC.[53] Early in the disease process, these tests occasionally can be shorter than normal, perhaps because of the presence of circulating factors that are already activated which would require less time for clot formation in vitro.[53]

In severe disease, the fibrinogen level can drop to 10–50 mg/dL but is decreased in only 23–71% of patients.[56] Fibrinogen is an acute phase reactant, and the protein increases in inflammatory conditions. Because many patients with DIC have underlying disease, their fibrinogen levels may have been initially elevated because of the acute phase response.

The D-dimer test to demonstrate the presence of fibrin-derived FDPs and therefore generation of both thrombin and plasmin is elevated in 93% of patients and provides good evidence supporting the diagnosis of DIC.[53] D-dimers, which are present only after FXIII stabilization of fibrin, confirm that activation of coagulation has taken place and stabilized thrombus is formed, but they are not specific for DIC. The D-dimer test can be positive in several other clinical conditions such as after surgery and in patients with renal disease or pulmonary emboli. Most laboratories use the D-dimer test, which has replaced a similar latex test for fibrin degradation products that fails to distinguish between plasmin degradation products of fibrinogen and fibrin (Chapter 36).

Some clinicians have recommended a test to demonstrate a decrease in AT. AT decreases in concentration early in the disease process as it combines with and inactivates thrombin and other serine proteases. AT is decreased in 89% of patients. Examination of the blood smear reveals the presence of schistocytes in 50% of patients. Schistocytes are produced as blood cells are forced through the fibrin webs that clog the microvessels. However, fragmented red blood cells rarely constitute >10% of the red cells on the peripheral smear.

The Scientific Standardization Committee of the International Society of Thrombosis and Hemostasis (ISTH) has developed a

★ **TABLE 34-11** Typical Laboratory Findings in Acquired Disorders of Hemostasis Associated with Bleeding

Test	DIC	Primary Fibrinogenolysis	Severe Liver Disease	Vitamin K Deficiency	Specific Factor Inhibitor	Lupus Anticoagulant[a]
Screening tests						
Platelet count	Dec	N	Dec	N	N	N
PT	Inc	Inc	Inc	Inc	N (inc with inhibitor against FVII)	N (occasionally inc)
APTT	Inc	Inc	Inc	Inc	Inc (except if inhibitor against FVII)	Inc
TT	Inc	Inc	Inc	N	N	N
Definitive tests						
Fibrinogen	Dec	Dec	Dec	N	N	N
D-dimer	Inc	N	N	N	N	N
Latex FDP test	Inc	Inc	N/Inc	N	N	N
Plasminogen	Dec	Dec	N/Dec	N	N	N
Fibrinopeptide A	Inc	N	N	N	N	N
Natural inhibitors						
Antithrombin	Dec	N	Dec	N	N	N
Protein C	Varies	N	Dec	Dec	N	N
Protein S	Varies	N	Dec	Dec	N	Varies
Miscellaneous						
Blood smear	Schistocytes	N	Macrocytes, target cells, acanthocytes	N	N	N

Inc = increased; Dec = decreased; N = normal; PT = prothrombin time; APTT = activated partial thromboplastin time; TT = thrombin time; FDP = fibrin degradation products

[a]Not a hemostatic disorder associated with bleeding but necessary to consider in the differential diagnosis.

diagnostic scoring system for DIC. This system uses simple, readily available coagulation testing in an algorithm that allows diagnosis and ongoing assessment of a patient with DIC[59,60] (Tables 34-12 ★ and 34-13 ★). With appropriate validation, these algorithms may help to standardize testing for DIC.

Other laboratory tests that could be abnormal in DIC but generally are not necessary for a diagnosis in most cases are the TT and serial specific factor assays for FV, FVIII, or prothrombin (to demonstrate decreased factor levels/increased consumption). The TT is prolonged because of the presence of FDPs and the decreased fibrinogen level and is abnormal in 58% of patients. Additional tests of thrombin generation include tests for fibrin monomers (soluble fibrin), FPA and -B, thrombin-AT complexes (TAT), and prothrombin fragment 1.2 (Chapter 36). These tests are all potentially useful parameters because they are very sensitive in detecting ongoing activation of coagulation, but presently are not routinely available in most hospital laboratories.

Therapy for DIC

The first step in the treatment for DIC is to eliminate its underlying cause if possible. After that, treatment is controversial. The acute form is often self-limited and disappears when the fibrin is completely lysed. Replacement therapy using platelets, red cells, cryoprecipitate, or fresh frozen plasma are used when indicated (patients with clear laboratory evidence of DIC and bleeding).[61] Low-molecular-weight heparins have been found helpful in some patients with strong clinical and laboratory evidence of DIC and predominant thromboembolic manifestations or in patients in whom replacement therapy fails to alleviate excessive bleeding and increase the level of clotting factors. Newer approaches to therapy are to replace the depleted physiologic inhibitors (AT, PC, tissue factor pathway inhibitor) with concentrates. Clinical trials are being conducted on some of these products. Under most circumstances, patients with DIC should not be treated with fibrinolytic inhibitors.

The chronic form of DIC is usually seen secondary to disseminated malignancy, in which case elimination of the precipitating event may be difficult. Heparin therapy sometimes is helpful if thrombosis is life threatening because it will stop intravascular fibrin formation.

★ TABLE 34-12 Diagnostic Algorithm for the Diagnosis of Overt Disseminated Intravascular Coagulation (DIC)

International Society on Thrombosis and Haemostasis

1. Risk assessment: Does the patient have an underlying disorder known to be associated with overt DIC?
   If yes: proceed; If no: do not use this algorithm.
2. Order global coagulation tests (platelet count, prothrombin time (PT), fibrinogen, soluble fibrin monomers or fibrin degradation products)
3. Score global coagulation test results.
   Platelet count *(>100 = 0; <100 = 1; <50 = 2)* ____
   Elevated fibrin-related marker (D-dimer, fibrin degradation products [FDPs])
   (no increase: 0; moderate increase: 2; strong increase: 3) ____
   Prolonged prothrombin time
   (<3 sec. = 0; >3 sec.– < 6 sec. = 1; >6 sec. = 2) ____
   Fibrinogen level
   (>1.0 gram/L = 0; <1.0 gram/L = 1) ____
4. Calculate score ____
5. If ≥5: compatible with overt DIC; repeat scoring daily
   If <5: suggestive (not affirmative) for nonovert DIC; repeat next 1–2 days.

Toh CH, Hoots WK, SSC on Disseminated Intravascular Coagulation of the ISTH. The scoring system of the Scientific and Standardisation Committee on Disseminated Intravascular Coagulation of the International Society on Thrombosis and Haemostasis: a 5-year overview. *J Thromb Haemostasis.* 2007;5: 604–606.

★ TABLE 34-13 Template for Scoring System for Nonovert DIC

International Society on Thrombosis and Haemostasis

1. Risk assessment: Does the patient have an underlying disorder known to be associated with DIC?
   Yes = 2; no = 0
2. Major criteria

Platelet count	$>100 \times 10^9$/L = 0	$<100 \times 10^9$/L = 1
	+	
Rising = −1	Stable = 0	Falling = 1
PT		
Prolongation	<3 sec. = 0	>3 sec. = 1
	+	
Falling = −1	Stable = 0	Rising = 1
Soluble fibrin		
Or FDPs	Normal = 0	Raised = 1
	+	
Falling = −1	Stable = 0	Rising = 1

3. Specific criteria

Antithrombin	Normal = −1	Low = 1
Protein C	Normal = −1	Low = 1
Thrombin/AT (TAT) complexes	Normal = −1	Low = 1
Other	Normal = −1	Abnormal = 1

4. Calculate score
   Specific score interpretations have not been developed.

☑ CHECKPOINT 34-8

a. Why is thrombocytopenia usually present in a patient with DIC?

b. Which hemostasis laboratory screening tests (PT and APTT), if any, will the following affect:

Decreased FV

Decreased FVIII

Decreased fibrinogen

Decreased antithrombin

c. Which laboratory test results would distinguish DIC from hemophilia A?

It must be administered with caution, however, because fatal bleeding has occurred with its use.

Primary Fibrinogenolysis

This syndrome is sometimes referred to as *primary fibrinolysis*, but this is technically inaccurate because the proteolytic action of plasmin is on fibrinogen, not fibrin. **Primary fibrinogenolysis** is a condition clinically similar to DIC but requires differentiation so that proper treatment can be instituted. In primary fibrinogenolysis, plasminogen becomes inappropriately activated to plasmin without concomitant thrombin generation. Plasmin then circulates and, if it overwhelms the antiplasmin inhibitors, degrades fibrinogen; factors V, VIII, and XIII; and other coagulation factors (and other proteins). An acquired deficiency of the proteins eventually develops and leads to bleeding symptoms that resemble DIC.

Similar pathologies can cause both DIC and primary fibrinogenolysis, but liver disease is one of the most common triggers of primary fibrinogenolysis. Differentiating the two conditions with laboratory tests is sometimes difficult. Patients with primary fibrinogenolysis can have an abnormal PT, APTT, TT, fibrinogen assay, and increased fibrin(ogen) degradation products but will have normal D-dimer and fibrin monomer tests because stabilized fibrin is not formed. The platelet count is typically normal in primary fibrinogenolysis, whereas a decreased platelet count is consistent with DIC.[62] The level of fibrinopeptide A is normal in primary fibrinogenolysis but is elevated in DIC.[63] See Table 34-11 for a comparison of laboratory test results in these two conditions with other acquired hemostatic disorders.

Therapy for primary fibrinogenolysis is epsilon aminocaproic acid (EACA, Amicar) or tranexamic acid (TXA); both prevent excessive plasmin activation. They occupy plasminogen's lysine-binding sites, thus blocking activation by tPA. These drugs can cause thrombotic complications in the microcirculation and therefore are dangerous if administered to patients with DIC; consequently, the diagnosis of DIC needs to be excluded before these drugs are given.[63,64]

Liver Disease

Liver disease affects all hemostatic functions. Most hemostatic proteins (those involved in fibrin formation, fibrinolysis, as well as hemostatic inhibitors) are synthesized by the liver (Web Table 34-1; Chapter 32). The liver macrophages play a major role in the removal of activated factors, products of activation such as the fibrinopeptides, fibrin degradation products, and plasminogen activators. A diseased liver diminishes these functions.[65]

Laboratory test results on a patient with liver disease can resemble those obtained from a patient with DIC. Differentiating the two conditions can be a difficult task for the physician. Refer to Table 34-11 for a comparison of the results of laboratory testing. The decreased production of proteins involved in fibrin formation can prolong all screening coagulation tests including the PT, APTT, and TT. The fibrinogen concentration is usually normal but can stabilize in the lower range of the reference interval. An abnormal fibrinogen molecule that has an increased content of sialic acid and can cause defective clot formation can be synthesized.

The platelet count can be decreased for several reasons, including hypersplenism (backing up of the portal blood supply when it is unable to enter the liver), alcohol toxicity of the bone marrow, decreased thrombopoietin production, and consumption of the platelets if DIC is also present.

FDPs are increased because the liver cells are unable to remove them from the circulation. Incomplete removal of plasminogen activators can result in systemic formation of plasmin and subsequent proteolysis of fibrinogen, contributing to the increase of FDPs. Excess fibrin or fibrinogen degradation products can impair blood coagulation and result in platelet dysfunction. The D-dimer test is usually normal and can be one way to differentiate DIC from liver disease.

Clinical bleeding is minimal except in severe liver disease when ecchymoses, hematomas, and epistaxis can occur. Bleeding from local lesions in the GI tract is common. Therapy involves the use of replacement products as needed.

Vitamin K Deficiency

Hepatic cells need vitamin K (VK) to complete the post-translational alteration of factors II, VII, IX, X, PC, and PS (Chapter 32). In the absence of VK, the hepatic cells synthesize precursor proteins, but because γ-carboxyglutamic acid residues are absent, the calcium-binding sites are nonfunctional. Deficiency of VK results in induced functional deficiencies of all of these proteins. If the level of functional proteins falls below 30 U/dL, bleeding symptoms can result, and the PT and/or the APTT is prolonged.

Sources of VK are green, leafy vegetables and synthesis by bacteria in the GI tract. Symptomatic VK deficiency in newborns results in **vitamin K deficiency bleeding (VKDB)**, formerly called **hemorrhagic disease of the newborn (HDN)**.[66] This deficiency can be seen in the first days of life. Because their livers are still immature, synthesis of the VK-dependent factors in newborns is 30–50% of adult levels.

VKDB is broken into three subtypes: early, classic, and late. Early VKDB occurs primarily in infants of mothers who have been on drugs that interfere with VK activity or metabolism, such as anticonvulsants, and usually occurs within the first 24 hours after birth. Classic VKDB occurs between days 1 and 7 and is largely prevented by prophylactic vitamin K administration at birth. Late VKDB occurs between day 8 and 6 months of age[66] and is more prevalent in breast-fed babies because human milk contains less vitamin K than does cow's milk. Infants with liver disease can also be susceptible to VKDB.

Manifestations of VKDB are bleeding in the skin or from mucosal surfaces, circumcision site, generalized ecchymoses, large intramuscular hemorrhages, and (rarely) intracranial bleeds. In the laboratory, the PT and possibly the APTT are more prolonged than expected at this age. Specific factor assays for factors II, VII, IX, and X are markedly decreased. The BT and the platelet count are within the reference interval (Table 34-11).

VKDB is prevented in the United States by the now standard practice of administering vitamin K to all newborns. Although most states have laws requiring its administration, some do not.

Causes of VK deficiency in adults include malabsorptive syndromes such as sprue, obstruction of the biliary tract (because bile salts are necessary for absorption of all fat soluble vitamin including VK), ingestion of VK inhibitors (such as warfarin), and prolonged broad-spectrum antibiotic therapy that abolishes normal flora of the intestine. VK administration corrects the deficiency within 24 hours.

Acquired Pathologic Inhibitors

Acquired inhibitors of blood coagulation, also called **circulating anticoagulants**, develop pathologically in patients with certain disease states and in some who have no apparent underlying condition. Almost all are immunoglobulins, primarily IgG or more rarely IgM, and can be either alloantibodies or autoantibodies. Two types of inhibitors are described: those directed toward a single coagulation factor and the lupus anticoagulant (LA).

Inhibitors of Single Factors

Pathologic inhibitors against most coagulation factors have been reported. Alloantibodies arise in individuals with congenital factor deficiencies as a consequence of replacement therapy. Autoantibodies arise in individuals whose coagulation system was previously normal and can be associated with other conditions such as diseases or drugs, or are sometimes seen in patients who are otherwise healthy.[67] With the exception of antibodies to FVIII and FIX, they are extremely rare. These inhibitors are recognized because of their interference with or neutralization of clotting factor activity. The following discussion concentrates on the FVIII and FIX inhibitors.

Clinical Aspects. Inhibitors to FVIII and FIX are observed most often in association with the hemophilias. Approximately 15–20% of patients with severe hemophilia A and 1–3% of patients with severe hemophilia B develop alloantibodies to the respective deficient factor. Inhibitors are neutralizing alloantibodies that render the replacement factor inactive. Twenty-two percent of hemophilia A patients with the inversion mutation described earlier develop FVIII inhibitors. Most patients with inhibitors have severe hemophilia with very low coagulant and immunologic levels of the affected factor. All patients also have received replacement therapy for their factor deficiency, and most inhibitors develop within the first 50–100 exposure days to factor replacement although patients can develop an inhibitor at any time in their life. In hemophilia A patients, the antibody specificity is directed toward the coagulant antigen (VIII:Ag) only, not the VWF portion of the FVIII/VWF complex. Although most inhibitors develop in severe or moderate forms of hemophilia, they also can be found in some patients with mild forms of hemophilia A.[68]

FVIII inhibitors (autoantibodies) can also be found in nonhemophilic patients (acquired hemophilia A). These inhibitors occasionally develop in otherwise healthy individuals who most often are older patients or females during or following a pregnancy. Disease states associated with FVIII inhibitors include autoimmune and lymphoproliferative diseases as well as multiple myeloma.[67] Autoantibodies to other clotting factors can appear in similar circumstances as the FVIII inhibitors.

The clinical course of patients with acquired inhibitors is variable but can resemble that of patients with severe hemophilia. These inhibitors result in fatal consequences in 10–20% of cases. In patients with congenital hemophilia who have not received therapy for 1 to 2 years, the antibody level often decreases, but an anamnestic response can be seen within 2 to 4 days after re-exposure. The antibody can also disappear spontaneously.

Laboratory Evaluation. Laboratory test results in patients with FVIII or FIX inhibitors resemble those in patients with severe factor deficiencies. The APTT is markedly prolonged, and other screening tests are normal (Table 34-11). Mixing studies (1:1 patient plasma with normal plasma) can be performed as a screening procedure to distinguish between a true factor deficiency and an inhibitor (Chapter 36). If an inhibitor is present, the test on the mixture will only partially correct or remain prolonged. In the presence of a low level of inhibitors, the test will correct initially but will show prolongation after incubating at 37°C for 1 or 2 hours. Assays for specific inhibitors can then be performed (Chapter 36).

Therapy for Factors VIII and IX Inhibitors. The type of therapy used for patients with FVIII inhibitors depends on whether the patient is a low or high responder and on the inhibitor's titer. Low responders (25% of hemophiliacs with inhibitors) are patients with low titer antibodies that do not rise after further exposure to FVIII. Patients with inhibitors that rise markedly with further exposure to FVIII (anamnestic response) are known as high responders (~75% of hemophiliacs with inhibitors). Large amounts of FVIII that function by "overwhelming" the antibody can be administered successfully to low responders to treat or prevent clinical bleeding. If human FVIII cannot be used because the inhibitor level is too high, FIX complex products can be used in an attempt to bypass the need for FVIII. This approach is sometimes referred to as ***f**actor **e**ight **i**nhibitor **b**ypassing **a**ctivity (FEIBA)*. FIX complex concentrates (i.e., PCC) contain prothrombin and factors VII, IX, and X. The mechanism of bypass activity of these concentrates remains unclear, but it has been suggested that their content of activated VK-dependent factors (IIa, Xa, trace amounts of VIIa and IXa) probably promote thrombin generation in vivo.[36] Recombinant FVIIa is another bypass agent effective in the treatment of hemophilia patients with inhibitors.[36] Recombinant FVIIa works by activating FX and bypassing the need for FVIII or FIX in the formation of fibrin and thus is equally effective in both hemophilia A and B patients with inhibitors.

Lupus Anticoagulant

The second type of immunoinhibitor of major clinical importance is the **lupus anticoagulant (LA)**, so called because it was first discovered in patients with systemic lupus erythematosus (SLE). Approximately 6–16% of patients with SLE develop LA. The term *lupus anticoagulant* is a misnomer because LA is more frequently encountered in patients without lupus and has been associated with a variety of other autoimmune diseases, neoplasias, certain infections, and the administration of drugs, such as chlorpromazine or procainamide, as well as in apparently normal individuals. However, many people still call it the *LA* or *lupuslike anticoagulant*.

LAs are autoantibodies that interact with the phospholipid surfaces of the reagents used in the APTT test (and occasionally the PT), prolonging the test results. They are part of a family of antibodies called *antiphospholipid antibodies* (*APLs*). LAs are usually discovered when an unexpectedly prolonged APTT and sometimes PT are found while performing routine coagulation studies. However, the LA is a laboratory phenomenon. Although laboratory testing suggests defective hemostasis, most patients do not in fact bleed but rather tend to be hypercoagulable. The pathophysiology, clinical aspects, and laboratory evaluation of LA/APL are discussed in Chapter 35 with other disorders of hypercoagulability.

CASE STUDY (continued from page 706)

A TT was performed on Scott, and the results were within the laboratory's reference interval. Mixing studies were performed. Scott's plasma was mixed with normal plasma, and the APTT was repeated on the mixture. The result of the APTT on the mixture was 36 sec.

8. What do the results of the APTT on the mixture of Scott's plasma with normal plasma indicate?
9. What test should be performed next?
10. If a FVIII assay were as done with results of <1 U/dL, what molecular studies should be done?
11. What therapy is indicated for this patient?
12. What complications from the therapy are possible?

FLOW CHARTS

A flow chart that outlines reflex testing procedures that the laboratory can follow to investigate abnormal hemostatic screening tests is included in this text's Companion Resources (Web Figure 34-2).

HEMOSTASIS IN THE NEWBORN

Hemostasis is an evolving process and is age dependent (i.e., it develops or evolves during the process of fetal development and changes with gestational age).[69] Blood coagulation studies in the newborn present special challenges to the laboratory. At birth, hepatic synthesis of several of the clotting proteins is at a lower level than in normal adults, and this makes diagnosing some inherited and acquired hemostatic abnormalities difficult. Some proteins do not reach adult levels until 6–12 months of age. Laboratory screening tests are prolonged (relative to adult normal values) and depend on the child's age and the presence of accompanying diseases.[70] These factors influence the interpretation of the laboratory tests.

Obtaining an adequate blood sample is of utmost importance but technically extremely difficult. Even with maximum attention to quality control, spuriously altered results are possible. A venous sample is preferred to capillary or arterial blood. Obtaining the sample from an indwelling catheter should be avoided because of the danger of heparin contamination.

Adding anticoagulant to the syringe allows one to draw blood more slowly while minimizing the risk of clotting. The amount of anticoagulant must be reduced when the infant's hematocrit is above 55% because of the reduced plasma volume.

Because the total volume of blood in newborns is only 250–350 mL, efforts must be made to minimize the amount of sample drawn (Chapter 36). Some suggestions for minimizing the amount of plasma needed for newborn testing are eliminating duplicate testing when performing the PT and APTT or running several factor assays using diluted plasma rather than the screening tests.[71] Microadaptations of several routine procedures, using 5–40 mcL of plasma, have been developed.[72]

Normal Hemostasis in the Newborn

Platelets and the proteins of the coagulation and fibrinolytic systems are first detected in fetuses at 10–11 weeks of gestation. See Table 34-14 ★ for expected values for various laboratory tests for preterm and term infants at birth compared with results in older children and adults as well as the ages when adult levels of the proteins are reached.

Platelet counts reach adult levels by 27 weeks of gestation and, therefore, should be in the normal adult reference interval (and size—i.e., mean platelet volume [MPV]) at birth. As with adults, platelet counts of $<100 \times 10^9$/L should be considered abnormal. Counts of $100–150 \times 10^9$/L are considered borderline and should be repeated. Platelet structure viewed by electron microscopy appears normal, but the dense body content of serotonin and ADP is <50% of adult levels.[69] Decreased platelet aggregation (relative to adult "normal") with

★ **TABLE 34-14** Laboratory Tests in Preterm and Term Infants as Compared with Those of Adults and Older Children

Test	Preterm Infant (28–31 weeks) (Day 1)	Preterm Infant (32–36 weeks) (Day 1)	Term Infant (37–41 weeks) (Day 1)	Adults and Older Children	Age Adult Level Reached
Platelet count, $\times 10^9$/L	150–430	150–430	174–456	150–450	Before birth
Platelet aggregation	Abnormal	Abnormal	Abnormal	Normal	One month
PT, sec	14.6–16.9[a]	10.6–16.2[a]	10.1–15.9[a]	10.8–13.9[a]	Comparable at birth
APTT, sec	80–168[a]	27.5–79.4[b]	31.3–54.5[a]	26.6–40.3[a]	By 6 months
TT, sec		19.0–30.4[b]	23.5 ± 2.38[c]	19.7–30.3[b]	At birth
FDP		Normal	Normal	Normal	Before birth

PT = prothrombin time; APTT = activated partial thromboplastin time; TT = thrombin time; FDP = fibrin degradation products

[a]Andrew M, Paes B, Johnston M. Development of the hemostatic system in the neonate and young infant. *Am J Ped Hem/Onc.* 1990;12(1):95–104.
[b]Andrew M, Paes B, Milner R et al. Development of the human coagulation system in the healthy premature infant. *Blood.* 1988;72:1651–657.
[c]Andrew M, Paes B, Milner R et al. Development of the human coagulation system in the full-term infant. *Blood.* 1987;70:165–72.

low levels of ADP and with collagen, epinephrine, and thrombin are found at birth but normalize within several weeks. Platelet agglutination with ristocetin, on the other hand, is increased in newborns and must be considered when diagnosing VWD. Plasma concentration of VWF and the proportion of high-molecular-weight multimers are increased in newborns (newborns have low levels of the VWF cleaving protease ADAMTS-13).[69] This likely explains the enhanced agglutination with ristocetin. Drugs that affect platelet function such as aspirin when taken by the mother also influence hemostasis in the fetus.

Concentrations of the coagulation proteins for term and preterm infants, older children, and adults are in Table 34-15 ★. Multiple reference intervals are required because the hemostasis system is evolving. The fibrinogen group of factors is at normal adult levels at birth. The concentrations of the proteins of the prothrombin and contact factor groups are decreased at birth because of newborn liver immaturity. They reach adult levels at varying times (Table 34-15). The levels of these clotting factors usually are not low enough to affect hemostasis unless a stressful situation is present. VWF, on the other hand, is increased at birth and gradually decreases over the first 6 months of life.[73]

Natural inhibitors that the liver synthesizes are also decreased in newborns (Table 32-15). Adults with levels of AT equivalent to that of infants are considered at risk for thrombosis. Infants do not have this problem because the procoagulant proteins that are inactivated by AT are also decreased. Levels of PC are very low at birth and remain decreased during the first 6 months of life. Total PS is also decreased, but functional activity is similar to that in adults because all PS is present in the free (active) form because of the absence of C4BP.[69] Plasminogen levels in infants are decreased, and FDPs are similar to adult values.

The decrease in hemostatic factors in term and preterm infants affects coagulation tests (Table 34-12). The PT is prolonged ~3 seconds compared with that of adults because of the low levels of factors II, VII, and X. However, a PT of >17 seconds should be considered abnormal in the newborn.[74] Values in the adult reference interval are usually achieved in 3–4 days.

★ **TABLE 34-15** Levels of Hemostatic Proteins in Preterm and Term Infants as Compared with Adults and Older Children

Protein	Preterm Infants[a] (27–31 Weeks) (U/dL) (Day 1)	Term Infants (38–41 Weeks) (U/dL) (Day 1)	Adults and Older Children (U/dL)	Age Adult Level Reached
Coagulant proteins—fibrinogen group				
Fibrinogen, mg/dL	256 ± 70	283 ± 116	278–122	Before birth
Factor V	65 ± 22	72 ± 35	62–150	Before birth
Factor VIII	37–126	50–178	50–149	Before birth
Factor XIII				
A subunit	32–108	27–131	55–155	5 days
B subunit	35–127	30–122	57–137	5 days
Coagulant proteins—prothrombin group				
Factor II (prothrombin)	19–54	26–70	70–146	6 months
Factor VII	24–76	28–104	67–143	5 days
Factor IX	17–20	15–91	55–163	6 months
Factor X	25–64	12–68	70–152	6 months
Coagulant proteins—contact group				
Factor XI	11–33	10–66	67–127	After 6 months
Factor XII	5–35	13–93	52–164	After 6 months
Prekallikrein	15–32	18–69	62–162	After 6 months
HMWK	19–52	6–102	50–136	1 month
Fibrinolytic protein				
Plasminogen	112–248[a]	195 ± 70	336 ± 88	6 months
Naturally occurring inhibitors				
Antithrombin	20–38	63 ± 24	105 ± 26	3 months
Protein C	12–44	35 ± 18	96 ± 32	After 6 months
Protein S	14–38	36 ± 24	92 ± 32	3 months
Heparin cofactor II	0–60	10–93	96 ± 30	6 months

Andrew M, Paes B, and Johnston M. Development of the hemostatic system in the neonate and young infant. *Am J Ped Hem/Onc.* 1990;12(1):95–104 except as noted ([a]).

[a]Andrew M, Paes B, Milner R et al. Development of the human coagulation system in the healthy premature infant. *Blood.* 1988;72:1651–657.

The APTT is also prolonged ~2–3 seconds in term infants and can be significantly prolonged in preterm infants. This test depends on the factors of the intrinsic system and is particularly sensitive to the contact factors. Results of this test are also highly dependent on the reagent used.[70,73] Adult levels can be reached in 4–6 months.[74] The thrombin clotting time is abnormal although the fibrinogen level is normal because of the presence of a distinct fetal fibrinogen molecule with altered function. The thrombin time becomes normal within a few days after birth.

In general, hemostatic values in preterm infants differ from adults as discussed. These values also differ from term infants but erratically so; however, by 6 months of age, their values are comparable to those of term infants.[75]

Common Bleeding Disorders in the Neonate

Although the coagulation systems of the "well" term and preterm infants show low levels of many procoagulant, anticoagulant, and fibrinolytic proteins, hemostasis is usually functionally balanced, and neither thromboses nor hemorrhages occur. However, abnormalities of hemostasis are present in ~1% of newborns. The classification of the most common problems depends on whether the child is considered sick or well. Sick infants include those with prematurity, perinatal infection, respiratory distress syndrome, metabolic derangements, and/or birth asphyxia. Hemostatic abnormalities in babies considered sick are most commonly either DIC, isolated platelet consumption independent of a decrease in clotting factors, or liver failure.

The most common abnormalities of hemostasis in well babies are immune thrombocytopenia, vitamin K deficiency, hemophilia, and bleeding from a localized vascular lesion. Diagnosing some hereditary bleeding disorders in the neonatal period is difficult, particularly mild or moderate deficiencies of FIX and VWD. Severe forms of FVIII or FIX deficiencies are easier to diagnose. Early onset vitamin K-deficiency bleeding (within the first 24 hours of life) is usually the result of placental transfer of maternal drugs that inhibit vitamin K activity in the baby, including Dilantin or other anticonvulsants, antibiotics, and oral anticoagulants.

Bleeding manifestations in babies with DIC are similar to those in other patients with the syndrome and include bleeding from puncture sites, the GI tract, and other locations. The PT and APTT are markedly prolonged, and thrombocytopenia is present in symptomatic babies.

One of the most common causes of death in premature infants is intracranial hemorrhage. Many of these are patients who have severe respiratory distress syndrome or a familial bleeding diathesis (hemophilia or another hereditary coagulation deficiency).

Thrombosis can also occur in infants, particularly those with indwelling catheters, those born to diabetic mothers, and those with predisposing medical conditions (e.g., asphyxia, infection, respiratory distress syndrome). Neonatal hypercoagulability can also be seen in infants with a hereditary thrombophilia (Chapter 35).

Summary

This chapter discusses the conditions associated with abnormal secondary hemostasis encompassing those in which fibrin is formed too slowly or poorly or fibrinolysis proceeds too rapidly so that excessive bleeding results. It also discusses the unique hemostatic status of newborns.

Abnormal fibrin formation occurs in patients with mutations of the genes that code for the circulating procoagulant proteins and result either in decreased synthesis or abnormal function of the protein. The most widely known of these disorders are X-linked deficiencies of FVIII and FIX, called the *hemophilias*. von Willebrand factor is complexed with FVIII in the circulation and when deficient can result in the most common bleeding disorder, von Willebrand disease. More unusual deficiencies of the remaining coagulation factors and of components of fibrinolysis can also result in excessive bleeding. However, in the case of factor XII, prekallikrein, and high-molecular-weight kininogen, no clinical bleeding symptoms are present in spite of abnormal in vitro laboratory test results. Some patients with deficiencies of these three components may have an increased risk for thrombosis, thought to be secondary to impaired fibrinolysis. Laboratory screening tests and specific factor assays establish the diagnosis in disorders associated with coagulant protein deficiencies.

Newborns are at higher risk for bleeding and can have prolonged PTs and APTTs until the vitamin K-producing intestinal bacteria and liver production of the various coagulation factors are established.

Review Questions

Level I

1. A patient who has a deficiency of a clotting factor could have: (Objective 1)
   A. inherited an abnormal gene from a parent
   B. acquired the deficiency because of another disease present
   C. decreased amount of the particular factor in the blood
   D. all of the above
2. Why do patients who have deficiencies of clotting factors usually have abnormal bleeding? (Objectives 1, 2)
   A. Fibrin formation is slower and less effective than normal.
   B. Platelets do not aggregate normally.
   C. Fibrin is formed too fast and in too large a quantity.
   D. Fibrin is broken down as fast as it is formed.
3. What clotting factor is deficient in a patient with hemophilia A? (Objective 4)
   A. FVII
   B. FVIII
   C. FIX
   D. FXIII

Use the following case study to answer questions 4–6.

An 18-year-old female bled profusely following extraction of a tooth. She had a history of sporadically increased menstrual bleeding and nosebleeds. She had had an appendectomy at age 10 with no unusual bleeding. A workup in the coagulation laboratory showed the following:

Laboratory Test	Patient Results	Laboratory Reference Interval
Platelet count	312 × 10^9/L	150–440 × 10^9/L
Bleeding time	9.5 minutes	2–9 minutes
Closure time	Increased	
Prothrombin time	11.5 sec	10–12 sec
Activated partial thromboplastin time	38.0 sec	23–36 sec
FVIII assay	20 U/dL	50–150 U/dL
FIX assay	102 U/dL	50–150 U/dL
Platelet aggregation studies	Normal: ADP, collagen, epinephrine Abnormal: ristocetin	

4. Which laboratory tests are outside their reference interval? (Objective 2)
   A. all tests shown
   B. platelet aggregation with ristocetin, activated partial thromboplastin time, FVIII assay
   C. platelet count, prothrombin time, FIX assay
   D. prothrombin time, activated partial thromboplastin time, FIX assay
5. The most probable cause of this patient's bleeding is: (Objectives 3, 10)
   A. vascular disorder
   B. FIX deficiency
   C. von Willebrand disease
   D. disseminated intravascular coagulation
6. What laboratory test that is abnormal in this patient is different from that of a patient with hemophilia A? (Objectives 5, 6)
   A. FVIII assay
   B. FIX assay
   C. platelet aggregation studies with ADP
   D. platelet aggregation studies with ristocetin
7. What result of the platelet count would you expect in a patient with hemophilia A? (Objective 5)
   A. normal
   B. increased
   C. decreased
   D. unpredictable
8. Which is/are characteristic/s of a patient with DIC? (Objective 8)
   A. a prolonged PT
   B. a prolonged APTT
   C. a decreased platelet count
   D. all of the above
9. What is the cause of disseminated intravascular coagulation (DIC)? (Objective 8)
   A. an inherited deficiency of FX
   B. a reaction to another disease that causes the hemostatic system to become activated
   C. a deficiency of vitamin K
   D. an antibody to FVIII

10. Which result would be expected in a newborn infant? (Objective 9)
    A. a shorter APTT test than that in an adult
    B. a longer APTT test than that in an adult
    C. a lower platelet count than that in an adult
    D. a higher platelet count than that in an adult

Level II

1. Referring to the case study for Level I questions 4–6, the results of the platelet aggregation studies indicate that the patient has a defect in which of the following? (Objective 4)
    A. FVIII
    B. platelet adhesion
    C. fibrinolysis
    D. intrinsic system of fibrin formation
2. What is the usual inheritance pattern of von Willebrand disease? (Objective 3)
    A. autosomal dominant
    B. autosomal recessive
    C. X-linked recessive
    D. not inherited, usually acquired
3. Which of the following is characteristic of type I von Willebrand disease? (Objective 4)
    A. decreased amounts of large multimers of VWF
    B. increased amounts of large multimers of VWF
    C. decreased amounts of all VWF multimers
    D. decreased amounts of small VWF multimers only
4. Which laboratory procedure analyzes VWF qualitatively for abnormalities of the molecular structure? (Objective 4)
    A. ristocetin-induced platelet aggregation
    B. FVIII assay
    C. SDS-page gel electrophoresis
    D. activated partial thromboplastin time
5. What is the cause of von Willebrand disease? (Objective 3)
    A. genetic mutations in the FVIII gene
    B. genetic mutations in the VWF gene
    C. genetic mutations in the glycoprotein Ib gene
    D. exposure to dyes and chemicals
6. In which of the following patients would the presence of delayed bleeding and deep muscular hematomas be most likely? (Objectives 1, 5)
    A. a patient with FVIII deficiency
    B. a patient with FXII deficiency
    C. a patient who is heterozygous for FV deficiency
    D. a patient with dysfibrinogenemia
7. In which of the following diseases would you most likely find an abnormal prothrombin time? (Objectives 4, 6)
    A. FVIII deficiency
    B. FIX deficiency
    C. disseminated intravascular coagulation
    D. prekallikrein deficiency
8. Which of the following is true concerning acquired circulating pathologic inhibitors to single coagulation factors? (Objective 9)
    A. They do not cause bleeding symptoms.
    B. They cause the same symptoms in the patient as an inherited deficiency of the same factor.
    C. They are often found in patients with von Willebrand disease.
    D. They are antibodies to the phospholipid in the coagulation reagents.
9. Which of the following is true in the condition known as *disseminated intravascular coagulation* (*DIC*)? (Objective 7)
    A. FV and FVIII become increased in activity.
    B. Fibrinolytic activity is absent.
    C. The patient has a single coagulation factor deficiency.
    D. Fibrinogen and platelets become depleted.
10. What laboratory test is helpful in differentiating primary and secondary fibrino(geno)lysis? (Objective 9)
    A. thrombin time
    B. D-dimer test
    C. plasmin-antiplasmin complexes
    D. platelet count

Companion Resources

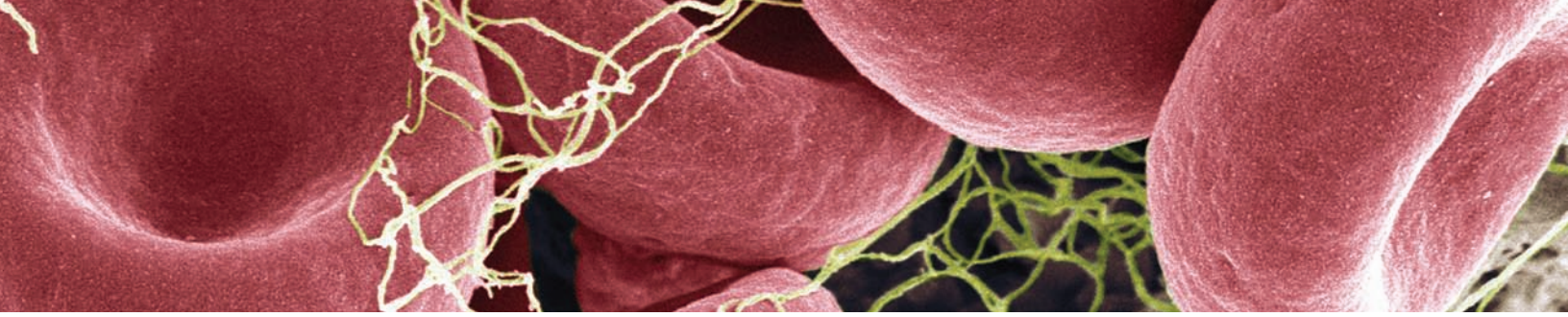
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Ann Rev Biochem*. 1998;67:395–424.
2. Mazurier C, Ribba AS, Gaucher C et al. Molecular genetics of von Willebrand disease. *Ann de Genet*. 1997;41:34–43.
3. Von Willebrand EA. Hereditary pseudohemophilia. *Haemophilia*. 1951;5:223–31.
4. Wagner DD, Ginsburg D. Structure, biology, and genetics of von Willebrand factor. In: Hoffman R, Benz EJ Jr, Shatill SJ et al., eds. *Hematology: Basic Principles and Practice*, 2nd ed. New York: Churchill Livingstone; 1995:1717–725.
5. Federici AB. Diagnosis of von Willebrand disease. *Hemophilia*. 1998; 4:654–60.
6. Vischer UM, de Moerloose P. von Willebrand factor: from cell biology to the clinical management of von Willebrand's disease. *Crit Rev Oncol Hematol*. 1999;30:93–109.
7. James, AH. More than menorrhagia: a review of the obstetric and gynaecological manifestations of bleeding disorders. *Haemophilia*. 2005;11:295–307.
8. Ginsburg D, Bowie EJW. Molecular genetics of von Willebrand disease. *Blood*. 1992;79:2507–519.
9. Veyradier A, Fressinaud E, Meyer D. Laboratory diagnosis of von Willebrand disease. *Int J Clin Lab Res*. 1998;28:201–10.
10. Hayward CPM, Harrison P, Cattane M et al. and the Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Platelet function analyzer (PFA-100®) closure time in the evaluation of platelet disorders and platelet function. *J Thromb Haemost*. 2006;4:312–19.
11. Favaloro EJ, Facey D, Henniker A. Use of a novel platelet function analyzer (PFA-100) with high sensitivity to disturbances in von Willebrand factor to screen for von Willebrand's disease and other disorders. *Am J Hematol*. 1999;62:165–74.
12. Caron C, Mazurier C, Goudemand J. Large experience with a factor VIII binding assay of plasma von Willebrand factor using commercial reagents. *Br J Haematol*. 2002;117:716–18.
13. Riddell AF, Jenkins PV, Nitu-Walley JC et al. Use of the collagen-binding assay for von Willebrand factor in the analysis of type 2M von Willebrand disease: a comparison with the ristocetin cofactor assay. *Br J Haematol*. 2002;116:187–92.
14. Sadler JE. A revised classification of von Willebrand disease. *Thromb Haemost*. 1994;71:520–25.
15. Sadler JE, Budde U, Eikenboom JCJ et al. Update of the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand factor. *J Thromb Haemost* 2006;4:2103–114.
16. Ginsburg D. Molecular genetics of von Willebrand disease. *Thromb Haemost*. 1999;82:585–91.
17. Tefferi A, Nichols WL. Acquired von Willebrand disease: concise review of occurrence, diagnosis, pathogenesis, and treatment. *Am J Med*. 1997;103:536–40.
18. van Genderen P, Boerjes RC, van Mourik JA. Quantitative analysis of von Willebrand factor and its propeptide in plasma in acquired von Willebrand syndrome. *Thromb Haemost*. 1998;80:495–98.
19. Sadler JE, Lillicrap D. von Willebrand disease: diagnosis, classification, and treatment. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:670–83.
20. Rogaev EI, Grigorenko AP, Faskhutdinova G et al. Genotype analysis identifies the cause of the "royal disease." *Science*. 2009;326:817.
21. Pavlovsky A. Contribution to the pathogenesis of hemophilia. *Blood*. 1947;2:185–91.
22. Marder VJ, Mannucci PM, Firkin BG et al. Standard nomenclature for factor VIII and von Willebrand factor: a recommendation by the International Committee on Thrombosis and Haemostasis. *Thromb Haemost*. 1985;54:871–72.
23. Greenberg CS, Orthner CL. Blood coagulation and fibrinolysis. In: Lee GR, Foerster J, Lukens J et al., eds. *Wintrobe's Clinical Hematology*, 10th ed. Baltimore: Williams & Wilkins; 1999:684–764.
24. Lillicrap D. Molecular diagnosis of inherited bleeding disorders and thrombophilia. *Semin Hematol*. 1999;36:340–51.
25. Carcao M, Moorehead P, Lillicrap D. Hemophilia A and B. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone/Elsevier; 2013:1940–960.
26. Roberts HR, Gray TF III. Clinical aspects of and therapy for hemophilia B. In: Hoffman R, Benz EJ Jr, Shatill SJ et al., eds. *Hematology: Basic Principles and Practice*, 2nd ed. New York: Churchill Livingstone; 1995:1678–685.
27. Tuddenham EGD, Collins PW, Yee TT. Clinical manifestations and therapy of inherited and acquired hemophilia. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:659–69.
28. Bretter DB, Kraus EM, Levine PH. Clinical aspects of and therapy for hemophilia A. In: Hoffman R, Benz EJ Jr, Shatill SJ et al., eds. *Hematology: Basic Principles and Practice*, 2nd ed. New York: Churchill Livingstone; 1995:1648–663.
29. Keeney S, Mitchell M, Goodeve A. The molecular analysis of haemophilia A: a guideline from the UK haemophilia doctors' organization haemophilia genetics laboratory network. *Haemophlia*. 2005;11:387–397.
30. Pruthi RK. Hemophilia: a practical approach to genetic testing. *Mayo Clin Proc*. 2005;80(11):1485–499.
31. Giangrande PLF. Management of pregnancy in carriers of haemophilia. *Haemophilia*. 1998;4:779–84.
32. Chuah MK, Nair N, VandenDriessche T. Recent progress in gene therapy for hemophilia. *Hum Gene Ther*. 2012;23:557–65.
33. Scott DW, Lozier JN. Gene therapy for haemophilia: prospects and challenges to prevent or reverse inhibitor formation. *Br J Haem*. 2011;156: 295–302.
34. Ponder KP. Gene therapy for hemophilia. *Curr Opin Hematol*. 2006;13: 301–07.
35. Tuddenham E. Gene therapy for haemophilia B. *Haemophilia*. 2012; 18(suppl 4):13–17.
36. Kessler CM, Mariani G. Clinical manifestations and therapy of the hemophilias. In: Colman RW, Marder VJ, Clowes AW et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2006:887–904.
37. Peyvandi F, Mannucci PM. Rare coagulation disorders. *Thromb Haemost*. 1999;82:1207–214.
38. Galanakis DK. Afibrinogenemias and dysfibrinogenemias. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:693–708.
39. Bolton-Maggs PHB, Perry DJ, Chalmers EA et al. The rare coagulation disorders—review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation. *Haemophilia*. 2004;10: 593–628.
40. Hanss M, Biot F. A database for human fibrinogen variants. *Ann N Y Acad Sci*. 2001;936:89–90. www.geht.org/databaseang/fibrinogen.
41. Neerman-Arbez M, deMoerloose P. Hereditary fibrinogen abnormalities. In: Kaushansky K, Lichtman MA, Beutler E et al., eds. *William's Hematology*, 8th ed. New York: McGraw-Hill; 2010:2051–68.

42. Mannucci P, Peyvandi F, Franchini M. Rare inherited coagulation disorders other than hemophilia. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:684–92.
43. Owren CA, Bowie EJW, Thompson JH. *The Diagnosis of Bleeding Disorders*, 2nd ed. Boston, MA: Little, Brown; 1975.
44. Millar DS, Elliston L, Deex P et al. Molecular analysis of the genotype-phenotype relationship in factor X deficiency. *Hum Genet*. 2000;106:249–57.
45. Peyvandi F, Menegatti M, Santagostino E et al. Gene mutations and three-dimensional structural analysis in 13 families with severe factor X deficiency. *Br J Haematol*. 2002;117:685–92.
46. Uprichard J, Perry DJ. Factor X deficiency. *Blood Rev*. 2002;16: 97–110.
47. Walsh, PN, Gailani D. Factor XI. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:233–41.
48. Duval C, Philippou H, Ariens RAS. Factor XIII. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013: 272–85.
49. CSL Behring received FDA approval of Cortifact™ for treatment of congenital factor XIII deficiency. King of Prussia, PA: CSL Behring; 2011. www.cslbehring.com/news-room/Corifact-FDA-Approval.
50. Jennings I, Kitchen S, Woods TA et al. Problems relating to the laboratory diagnosis of factor XIII deficiency: a UK NEQAS study. *J Thromb Haemost*. 2003;1:2603–608.
51. Katona E, Haramura G, Karpati L et al. A simple, quick one-step ELISA assay for the determination of complex plasma factor XIII (A_2B_2). *Thromb Haemost*. 2000;83:268–73.
52. Zhang B, Ginsburg D. Familial multiple coagulation factor deficiencies. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:709–14.
53. Bick RL. *Disorders of Thrombosis and Hemostasis: Clinical and Laboratory Practice*. Chicago: ASCP Press; 1992.
54. Colman RW. Contact activation pathway: inflammation, fibrinolytic, anticoagulant, antiadhesive and antiangiogenic activities. In: Colman RW, Marder VJ, Clowes AW et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2006:103–22.
55. Stewart DB, Francis CW, Marder VJ. Clinical disorders of fibrinolysis. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013: 715–22.
56. Levi M, de Jonge E, van der Poll T et al. Disseminated intravascular coagulation. *Thromb Haemost*. 1999;82:695–705.
57. Scherer RU, Spangenberg P. Procoagulant activity in patients with isolated severe head trauma. *Crit Care Med*. 1998;26:149–56.
58. Levi M, Feinstein DI, Colman RW et al. Consumptive thrombohemorrhagic disorders. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1178–95.
59. Levi M, Seligsohn U, Disseminated intravascular coagulation. In: Kaushansky K, Lichtman MA, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:2101–120.
60. Toh CH, Hoots WK on behalf of the SSC on Disseminated Intravascular Coagulation of the ISTH. The scoring system of the Scientific and Standarisation Committee on Disseminated Intravascular Coagulation of the International Society on Thrombosis and Haemostasis: a 5-year overview. *J Thromb Haemost*. 2007;5:604–606.
61. Baglin T. Disseminated intravascular coagulation: diagnosis and treatment. *Br Med J*. 1996;312:683–87.
62. Kernoff PBA, McNichol GP. Normal and abnormal fibrinolysis. *Br Med Bull*. 1977;33:239–44.
63. Grosset AMB, Rodgers GM. Acquired coagulation disorders. In: Lee GR, Foerster J, Lukens J et al., eds. *Wintrobe's Clinical Hematology*, 10th ed. Baltimore: Williams & Wilkins; 1999:1733–780.
64. Stewart DB, Marder VJ. Therapy with antifibrinolytic agents. In: Marder VJ, Aird WC, Bennett JS et al. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:918–30.
65. Eby CS, Caldwell SH. Hemostatic challenges in liver disease. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1481–490.
66. Zipursky A. Prevention of vitamin K deficiency bleeding in newborns. *Br J Haematol*. 1999;104:430–37.
67. Hay CRM. Acquired disorders of coagulation: the immune coagulopathies. In: Marder VJ, Aird WC, Bennett JS et al. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:723–37.
68. Key, NS. Inhibitors in congenital coagulation disorders. *Br J Haematol*. 2004;127:379–91.
69. Monagle P, Andrew M. Developmental hemostasis: relevance to newborns and infants. In: Nathan DGG, Orkin SH, Ginsburg D et al., eds. *Hematology of Infancy and Childhood*, 6th ed. Philadelphia: WB Saunders; 2003:121–68.
70. Montgomery RR, Marlar RA, Gill JC. Newborn hemostasis. *Clin Haematol*. 1985;14:443–60.
71. Monagle P, Ignjatovic V, Berry LR. Developmental hemostasis. In: Marder VJ, Aird WC, Bennett JS et al. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1411–23.
72. Johnston M, Zipursky A. Microtechnology for the study of blood coagulation system in newborn infants. *Can J Med Tech*. 1980;42:159–64.
73. Andrew M, Paes B, Milner R et al. Development of the human coagulation system in the full-term infant. *Blood*. 1987;70:165–72.
74. Hathaway W, Corrigan J for the Subcommittee. Normal coagulation data for fetuses and newborn infants. *Thromb Haemost*. 1991;65:323–25.
75. Andrew M, Paes B, Milner R et al. Development of the human coagulation system in the healthy premature infant. *Blood*. 1988;72:1651–657.



35 Thrombophilia

LYNNE WILLIAMS, PHD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *hypercoagulability*, *thrombophilia*, *thrombus*, and *thrombosis*.
2. Describe the physiological processes involved in hypercoagulability.
3. Explain how a thrombus becomes a thromboembolus.
4. Contrast a white thrombus and a red thrombus, and list the risk factors predisposing to the formation of each.
5. Define *deep vein thrombosis (DVT)*; explain how it is diagnosed and what clinical conditions can result from it.
6. Describe the role of heparin in the neutralization of activated coagulation factors by antithrombin.
7. Diagram the relationship of protein C (PC) and protein S (PS) to the coagulation pathway and explain why a deficiency of either might lead to a thrombotic tendency.
8. Explain why both molecular (antigenic) and functional assays should be performed for diagnosing antithrombin, PC, or PS abnormalities in a patient.
9. Describe activated PC (APC) resistance (APCR), and explain its contribution to thrombophilia.
10. List two side effects of heparin therapy and what hematology procedures should be monitored to prevent or limit these complications.
11. Contrast unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) including anticoagulant mechanisms and monitoring.
12. Discuss how oral anticoagulants such as Coumadin decrease a person's risk for thrombosis, and describe the best way to monitor oral anticoagulation.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Evaluate the utility of laboratory tests in the differential diagnosis of arterial and venous thrombotic disease.
2. Describe the role of microparticles in arterial and venous thrombosis.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

3. List four clinical manifestations suggestive of an inherited thrombophilia, and explain why many patients are not diagnosed.
4. Explain the differences between type I and type II deficiencies of protein C (PC), protein S (PS), and antithrombin; given values for antithrombin or PC and PS assays, determine the type of deficiency in a patient.
5. List two deficiencies that can cause warfarin- (Coumadin-) induced skin necrosis and give potential treatment options.
6. Justify performing both a clotting and molecular assay for diagnosis of activated PC resistance (APCR).
7. Describe the two most common mutations leading to hyperhomocysteinemia.
8. Explain how increased levels of prothrombin or fibrinogen might predispose to thrombosis.
9. Describe how deficiencies of plasminogen or plasminogen activator or increased levels of plasminogen activator inhibitor could lead to thrombosis.
10. Identify secondary disorders leading to thrombosis.
11. Describe and explain the treatment of a thrombotic episode.
12. Explain how the international normalized ratio (INR) has standardized prothrombin times.
13. Evaluate the use and monitoring of thrombolytic therapy in treatment of thrombosis.
14. Given a patient history and appropriate laboratory results, interpret the data to determine a probable diagnosis.

Key Terms

Activated protein C resistance (APCR)
Antiphospholipid antibody (aPL)
Antiphospholipid syndrome (APS)
Deep vein thrombosis (DVT)
Disseminated intravascular coagulation (DIC)
Dysfibrinogenemia
Embolus
Factor V Leiden (FVL)
Hemolytic uremic syndrome (HUS)
Heparin-induced thrombocytopenia (HIT)
Hypercoagulable
Hyperhomocysteinemia
Low-molecular-weight heparin (LMWH)
Microparticles (MP)
Pulmonary embolism (PE)
Red thrombi
Thromboembolism (TE)
Thrombolytic therapy
Thrombophilia
Thrombophlebitis
Thrombosis
Thrombotic thrombocytopenic purpura (TTP)
Thrombus
Unfractionated heparin (UFH)
White thrombi

Background Basics

Information in this chapter builds on concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the mechanisms of platelet activation and the functions of platelets in both primary and secondary hemostasis. (Chapter 31)
- Outline the coagulation cascade and the components contributing to fibrin formation. (Chapter 32)
- List the components and the function of the fibrinolytic system and the products produced by fibrinolytic action (FDPs). (Chapter 32)
- List the mechanisms that maintain normal physiologic control of hemostasis, particularly the inhibitors of both coagulation and fibrinolysis. (Chapter 32)

Level II

- Describe the mechanism of activation of serine proteases (complex formation on a phospholipid surface). (Chapter 32)
- Summarize the physiologic controls that limit activation of coagulation and fibrinolysis to the sites of vessel injury. (Chapter 32)
- Describe the method of activation and physiologic function of the inhibitors of coagulation and fibrinolysis. (Chapter 32)

CASE STUDY

We will address this case study throughout the chapter.

Andrea is a 37-year-old woman recently diagnosed with deep vein thrombosis (DVT) of the right leg. She is dehydrated and taking oral contraceptives. She is also taking Coumadin.

Consider possible factors contributing to Andrea's thrombosis and the laboratory tests that might be used to differentiate and diagnose the cause.

OVERVIEW

This chapter describes imbalances between procoagulant and anticoagulant processes in the hemostatic system that result in an increased tendency to form thrombi (thrombophilia). The chapter begins with a summary of the factors that influence thrombus formation in the arterial and venous blood vessels and the nature of the resulting thrombi that form in each side of the circulatory system. The chapter defines *thrombophilia* and describes the hereditary and acquired factors that contribute to it. The chapter discusses the pathophysiologic basis, clinical manifestations, and laboratory diagnosis for each thrombophilic factor with emphasis on the concept of thrombophilia as a

multifactorial disease process. For details on laboratory tests mentioned in this chapter, refer to Chapters 36 and 37. The chapter ends with a brief discussion of the available therapies for the thrombophilic patient.

INTRODUCTION

The human hemostatic system represents a balance between procoagulant or thrombogenic factors and anticoagulant and/or fibrinolytic activity. In a normal healthy individual, the two systems are balanced so that neither excessive bleeding nor clotting occurs. When the balance is tipped so that there is more procoagulant or clotting activity (or less anticoagulant activity), the person is said to be **hypercoagulable**. **Thrombosis** is the formation of a platelet and/or fibrin mass within a vessel, known as a **thrombus** (plural, *thrombi*). Many conditions can cause hypercoagulability and lead to thrombosis.

The three physiologic components responsible for hemostasis are also the sources of thrombogenic influences: the vascular endothelial cell, platelets, and coagulation proteins (Table 35-1 ★). The normal intact endothelium is antithrombogenic and does not activate platelets or blood coagulation factors. Damage to vascular endothelial cells can cause the release of prothrombotic substances (e.g., tissue factor, thromboxane [TXA_2], plasminogen activator inhibitor [PAI-1]) or loss of normally protective antithrombotic functions (e.g., thrombomodulin/protein C [PC] activation, heparan sulfate/antithrombin [AT] activation, nitric oxide, prostacyclin) (Figure 31-3). Circulating platelet agonists or interaction of platelets with exposed subendothelium can activate platelets. Tissue factor (TF) or collagen resulting from damaged vessels can activate procoagulant plasma proteins.

★ **TABLE 35-1** Physiologic Alterations Contributing to Thrombosis

Thrombogenic alterations

- Damage/activation of endothelial cells
- Exposure of subendothelium (loss of endothelial cells)
- Activation of platelets (circulating agonists, interaction with subendothelium)
- Activation of plasma coagulation proteins
- Inhibition of fibrinolysis
- Stasis

Loss of protective mechanisms

- Antithrombotic factors released from intact endothelium (prostacyclin, nitric oxide)
- Neutralization of activated coagulation factors by endothelial components (thrombomodulin, heparan sulfate)
- Inhibition of activated coagulation factors by naturally occurring plasma protease inhibitors (AT, TFPI, HCII)
- Degradation of coagulation factors by inhibiting proteases (APC)
- Dilution of activated clotting factors and disruption of platelet aggregates by unimpeded blood flow
- Breakdown of fibrin thrombi by the fibrinolytic system

AT = antithrombin; TFPI = tissue factor pathway inhibitor; HCII = heparin cofactor II; APC = activated protein C

Inadequate control of plasma coagulation can result from decreased activity of protective protease inhibitors or inadequate fibrinolysis. Additionally, stasis, or impaired blood flow, is thrombogenic. Normal unimpeded blood flow dilutes or removes activated clotting factors from their site of activation.

Thrombi can form in any part of the cardiovascular system, including the arteries, veins, heart, and microcirculation. Thrombi are composed of fibrin, platelets, and entrapped cells; the relative proportion of each component is influenced by hemodynamic factors and differs in arterial and venous thrombi. The thrombus is often incorrectly referred to as a *clot*; however, a clot is a mass that forms extravascularly (either in vitro or from bleeding into the tissues or body cavities). Thrombus formation can lead to two major pathologic events. The thrombus can enlarge, causing local obstruction of the blood vessel (*ischemia*) and resulting in the death of the tissue supplied by that vessel (*necrosis*). A piece of thrombotic material (an **embolus**) can break off from the thrombus and travel through the circulatory system, lodging at a distant site and obstructing blood flow in that tissue, causing ischemia and necrosis. This obstruction is termed an *embolism*. If the blockage originates from a thrombus, it is referred to as a **thromboembolism (TE)**.

THROMBUS FORMATION

Arterial Thrombi

Thrombi formed in arteries where blood flow is rapid are termed **white thrombi**. They are composed primarily of platelets and fibrin with relatively few leukocytes and erythrocytes. Arterial thrombi usually form in regions of disturbed flow, at sites of damage to the endothelium, often in areas of atherosclerotic plaques. Plaques are composed of extracellular lipids, fibrous connective tissue, and foam cells (e.g., lipid-filled macrophages, smooth muscle cells). Thrombosis is generally initiated by endothelial denudation of the plaque, exposing subendothelial thrombogenic material to the blood. Platelet activation and activation of plasma coagulation factors occur, resulting in the formation of fibrin. The activated platelets and fibrin interact to form a thrombus that can extend into the lumen of the vessel, eventually obstructing the artery and inducing ischemia. An embolus can break off and lodge in the small vessels of the heart or brain, causing myocardial or cerebral infarction (and tissue death).[1] In addition to endothelial cell loss, more subtle endothelial injury (e.g., exposure to endotoxin, hypoxia, cytokines such as interleukin-1 or TNF [tumor necrosis factor]) can also promote coagulation. Treatment for arterial thrombi usually involves platelet-inhibiting drugs (e.g., aspirin, clopidogrel, or ticlopidine) or thrombolytic therapy.

Traditional risk factors associated with an increased predisposition to atherothrombosis and the formation of arterial thrombi include hypercholesterolemia, hypertension, smoking, physical inactivity, obesity, and diabetes. Historically, up to one-half of all patients who developed a coronary thrombosis did not have any of these conventional risk factors. Consequently, there was interest in identifying additional risk markers that could predict an impending arterial thrombotic event. More recently recognized risk factors include hyperhomocysteinemia, elevated lipoprotein a (Lp[a]), and oxidized low-density lipoproteins (oxLDL).[2] Standard laboratory tests (prothrombin time [PT], activated partial thromboplastin time [APTT],

and thrombin time [TT]) are neither specific nor sensitive for predicting arterial thrombi. Thus, novel risk factors and laboratory tests have been investigated. Some additional tests that are often included in a thrombotic profile are hyperhomocysteinemia; elevated Lp(a); quantitative fibrinogen; D-dimer; PAI-1; C-reactive protein (CRP); and decreased tPA. None of these tests is specific for arterial thrombosis. Tests suggesting an increase in procoagulant activity (elevated fibrinogen, hyperhomocysteinemia) or decreased fibrinolytic activity (elevated PAI-1, decreased tPA) can reflect a thrombotic tendency but not specifically arterial thrombosis.

It is now well accepted that inflammation plays a major role in the initiation and progression of atherothrombosis.[2,3] Thus, inflammation can be linked to cardiovascular risk, and a number of different biomarkers of inflammation have been shown to be useful as predictors of preclinical atherothrombosis. Clinical data for CRP, fibrinogen, and serum amyloid A (acute-phase reactant proteins) strongly support a role for inflammation in atherothrombosis.[4,5] Of these, the assay for high sensitivity CRP (hsCRP) has emerged as the most useful.[2]

CRP is a typical acute-phase reactant with elevated serum levels occurring in response to acute injury, infection, or other inflammatory stimuli. The high sensitivity assay for CRP (hsCRP) is widely used as a biomarker for chronic vascular inflammation. CRP provides additional prognostic information to LDL cholesterol assays. Individuals with low LDL cholesterol levels but elevated CRP have a higher risk of developing future vascular disease than those with elevated LDL cholesterol but low levels of CRP.[6] Evidence of inflammation can be detected many years in advance of acute thrombosis in apparently healthy men and women, indicating that inflammatory processes occur early and precede vascular occlusion.[7]

Manifestations of arterial thrombotic disease include acute coronary syndromes (ACS)/coronary heart disease (CHD), ischemic stroke (a disturbance of cerebral function lasting >24 hours), and peripheral artery disease (PAD—affecting the lower extremities).

Venous Thrombi

Thrombi formed in the veins where blood flow is slower are termed **red thrombi**. They are primarily composed of red blood cells trapped in the fibrin mesh with relatively fewer platelets and leukocytes. Such thrombi usually form in regions of slow or disturbed blood flow, often in venous segments that have been exposed to direct trauma.

Although venous thrombi can occur in any vein in the body, they most commonly occur in the lower limbs. Thrombosis of the upper extremities (brachial, axillary or subclavian veins) constitute only 4–10% of reports of venous thrombosis.[8] Thrombosis of the superficial veins of the legs is usually benign and self-limiting (**thrombophlebitis**). This is primarily an inflammatory process triggered by mechanical or chemical irritants or in varicose veins with slow blood flow.[9] In contrast, involvement of the deep veins of the leg (**deep vein thrombosis [DVT]**) is more serious. Generally, distal thrombi localized to the deep veins of the calf are less serious (and often undergo spontaneous lysis) than those involving the proximal veins (popliteal, femoral, or iliac veins). DVT involving the calf are also less commonly associated with long-term disability. The symptoms of venous thrombosis can include localized pain, warmth, redness, and swelling. **Pulmonary embolism (PE)** refers to the presence of thrombi in the pulmonary arteries; it originates from lower limb DVT in >90% of cases. Embolization of a (deep vein) thrombus to the pulmonary circulation is a serious and potentially life-threatening complication of DVT. A strong association exists between finding a PE and the presence of venous thrombosis in the lower limbs; PE is detected in ~50% of patients with documented proximal DVT.[10] DVT and PE are thought to be different stages and clinical presentations of the same pathologic process, termed *venous thromboembolism (VTE)*. The incidence of DVT is 100–380 cases per 100,000 individuals per year,[11] and about 30% of patients with it experience a recurrence within 10 years.[12]

Like arterial thrombosis, venous thrombosis occurs when activation of blood coagulation exceeds the ability of the natural protective mechanisms (anticoagulants or inhibitors and the fibrinolytic system) to prevent fibrin formation (Table 35-1). Venous thrombosis is associated with the activation of plasma coagulation proteins initiated by vascular trauma or inflammation, the consequences of which are amplified by stasis. Venous stasis predisposes to local thrombosis by impairing the clearing of activated coagulation factors.

VTE can occur spontaneously or be provoked by recognizable clinical risk factors.[13] About 60% of VTE events occur in hospitalized and nursing home patients, suggesting that prophylactic treatment of certain patient groups may reduce the incidence of VTE.[14] Clinical risk factors that predispose to venous thrombosis include the following (risk factors 3–8 are discussed later in this chapter):

1. *Venous stasis* Venous return from the legs is enhanced by contraction of calf muscles, which act as a pump to propel blood from the legs toward the heart. Immobility (loss of the pumping action of the contracting muscles), venous obstruction, venous dilation, and increased blood viscosity (polycythemia, dysproteinemia, elevated fibrinogen) all predispose to venous thrombosis.
2. *Vessel wall damage* The vascular endothelium can be damaged by direct trauma (surgery, severe burns, severe trauma) and exposure to endotoxin, inflammatory cytokines (interleukin-1, TNF), thrombin, or hypoxia. Damaged endothelial cells synthesize tissue factor and PAI-1 and reduce thrombomodulin and tPA expression—changes that promote thrombogenesis[10] (Figure 31-3).
3. *Factor V Leiden (FVL) and activated PC resistance*
4. *Deficiency of circulating protease inhibitors* Antithrombin, PC, protein S [PS], heparin cofactor II (HCII).
5. *Elevated prothrombin levels* Prothrombin 20210.
6. *Antiphospholipid antibodies*
7. *Hyperhomocysteinemia*
8. *Decreased fibrinolytic activity* Fibrinolytic activity has been found to be lower in leg veins than in arm veins, which could partly explain the greater tendency for venous thrombosis to occur in the lower extremities.[15]
9. *Malignancy* The association between venous thrombosis and cancer has been attributed to procoagulant material released from the tumor cells or to complications of chemotherapy.
10. *Surgery* Surgery has been recognized as a major risk factor for VTE; certain surgical procedures have a higher risk than others.
11. *Miscellaneous* Other factors associated with increased risk of thrombosis include advanced age, obesity, pregnancy, use of oral contraceptives (OCs) and hormone replacement therapy, smoking, hypertension, hyperlipidemia, and a clinical history of

previous venous thromboembolism. Blood groups other than group O (e.g., A, B, AB) have been shown in some studies to have an increased risk of thrombosis,[16–20] perhaps the result of higher levels of von Willebrand factor (VWF) in blood groups A, B, and AB.[21]

The diagnosis of DVT can be difficult because similar clinical features and laboratory test results are found in other conditions. Clinical/laboratory diagnosis of venous thrombosis is generally accepted to be unreliable and objective tests are needed to confirm the diagnosis.[9] Objective tests using radiologic procedures to visualize the thrombus can help in identifying a thrombus. Venography, the traditional "gold standard" for diagnosing DVT, has largely been replaced by noninvasive venous compression ultrasonography (CUS), and spiral computerized tomography (s-CT). Likewise, pulmonary angiography has largely been replaced by computed tomography of the pulmonary arteries to confirm PE.[9,22] Blood tests for biologic markers of thrombin generation and fibrinolysis are often elevated in patients with DVT. Laboratory tests used in diagnosis include prothrombin fragment 1.2, fibrinopeptide A (FPA), thrombin-AT (TAT) complex, soluble fibrin monomer, D-dimer, tPA, and PAI-1. However, these tests are nonspecific and can be abnormal in a number of clinical conditions associated with excessive coagulation and/or fibrinolysis and thus have low specificity for DVT. An appropriately validated quantitative D-dimer assay (different assays have variable sensitivity and specificity) seems to have both high sensitivity and a negative predictive value; thus, a negative result can be used to rule out DVT.[23,24]

☑ CHECKPOINT 35-1

A patient has low levels of tPA and elevated levels of PAI-1. Why would this result in hypercoagulability in this patient?

Microparticles in Arterial and Venous Thrombosis

Microparticles (MPs) are cell-derived products that have been recognized as playing a key role in thrombus formation.[25–27] They are membrane-bound vesicles produced by blebbing of the cell membrane of the parental cell and are released in response to cell activation or apoptosis. They range in size from 100–1000 nM, and carry many of the antigens of the parent cell.[28]

Initially, MPs were recognized for having procoagulant potential, associated with the externalization of phosphatidyl serine, which is normally sequestered on the inner leaflet of the cell membrane, and/or expression of TF.[29] More recently, MPs have been recognized as playing a role in other physiologic processes, including inflammation, angiogenesis, and immune responses.[30] MPs have also been reported to promote anticoagulant functions and fibrinolytic responses, possibly at times counteracting their prothrombotic activity.[31]

MPs can be derived from a variety of cell types, including platelets, endothelial cells, erythrocytes, leukocytes, and cancer cells. Most of the MPs found in the circulation of healthy individuals are platelet derived. However, there are significant changes in the quantity and cellular origin of MPs in patients with arterial and venous thrombosis.[32] MPs from different cellular sources differ in their expression of phosphatidyl serine and/or TF, each of which contributes to the procoagulant properties of MPs.[27] Numerous studies have reported on the involvement of TF-expressing MPs in both arterial and venous thrombosis. However, there is still controversy on whether the MPs are truly a cause or are a consequence of thrombosis.[27]

The feasibility and utility of detecting and quantifying microparticles as biomarkers for thrombosis are under investigation.[27,33] Accurate detection of MPs is technically challenging, and a number of detection methods are being explored.[33] It is likely that evaluation of circulating MPs will eventually be added to the menu of laboratory tests used in the diagnosis of thrombosis and/or prediction of thrombotic risk.

THROMBOPHILIA

The two most common causes of death worldwide in the past decade were ischemic heart disease and stroke with 7 million and 6.2 million people, respectively, dying annually.[34] A congenital abnormality and/or an acquired alteration can cause thrombotic episodes. The proper treatment or prevention of thrombosis depends on careful clinical assessment and extensive testing procedures.

The term **thrombophilia** is used to describe any disorder either inherited or acquired associated with an increased risk of thrombosis. This increased risk of thrombosis is also referred to as *hypercoagulability*. Although thrombosis at a young age is probably the most important feature of inherited thrombophilia, many patients have their first episode later in life. Women with certain inherited thrombophilic abnormalities have an increased risk of pregnancy loss, preeclampsia, and **h**emolysis, **e**levated **l**iver enzymes, and **l**ow **p**latelet count (HELLP) syndrome (Chapter 20), whereas some acquired thrombophilic conditions are also associated with recurrent pregnancy loss.[35] Carriers of some inherited thrombophilic defects are prone to other thrombotic complications, such as vitamin K antagonist- (warfarin)-induced skin necrosis or heparin resistance. The thrombotic risk of a given hereditary predisposition or an acquired condition varies from low to modest to high risk of VTE (Table 35-2 ★).

An inherited thrombophilic risk factor places an individual at risk for but does not inevitably lead to thrombosis. Not all persons with thrombophilia experience a thrombotic event; many do not develop thrombosis unless some other (acquired) risk factor is present. Surgery, pregnancy, immobilization, estrogen therapy, obesity, trauma, and infection can initiate thrombotic events. These conditions can lead to thromboses in people with or without an inherited thrombotic tendency, so when a thrombotic episode occurs, a decision must be made as to whether it represents an isolated event or the patient has an underlying genetic predisposition. A thorough personal and family history can help in determining the likelihood of a genetic component. However, some affected family members may be asymptomatic, their condition discovered only after another family member is diagnosed. More than one hemostatic defect or abnormality significantly increases the risk for thrombosis.

★ **TABLE 35-2** Risk of Clinical Venous Thromboembolic Disease

	Low	Moderate	High	Excessive
Hereditary	Heterozygous APCR	Heterozygous AT deficiency	Double heterozygous APCR/ prothrombin 20210	Homozygous or double heterozygous AT, PC, PS
	Heterozygous prothrombin 20210	Heterozygous PC or PS deficiency	Homozygous prothrombin 20210	
	Heterozygous HCII		Homozygous factor V Leiden (APCR)	
	Sickle cell anemia			
	MTHFR C677T			
	Blood group A, B, AB			
	Dysfibrinogenemia			
Acquired	General surgery	Sepsis	Hip fracture	Mucin-secreting adenocarcinoma
	Oral contraceptives pregnancy	Surgery for malignancy	Total hip or knee replacement	
	Long trips	Prolonged immobilization	Acute promyelocytic leukemia	
	Elevated FVIII	Antiphospholipid Ab	TTP	
	Elevated Lp(a)	Myeloproliferative neoplasms		
	HIT	PNH		
		Major surgery/major trauma		

APCR = activated protein C resistance; AT= antithrombin; PC = protein C; PS = protein S; HCII = heparin cofactor II; MTHFR = methy ltetrahydro folate; Ab = antibody; TTP = thrombotic thrombocytopenia purpura; Lp(a) = lipoprotein A; HIT = heparin induced thrombocytopenia; PNH = paroxysmal nocturnal hemoglobinuria

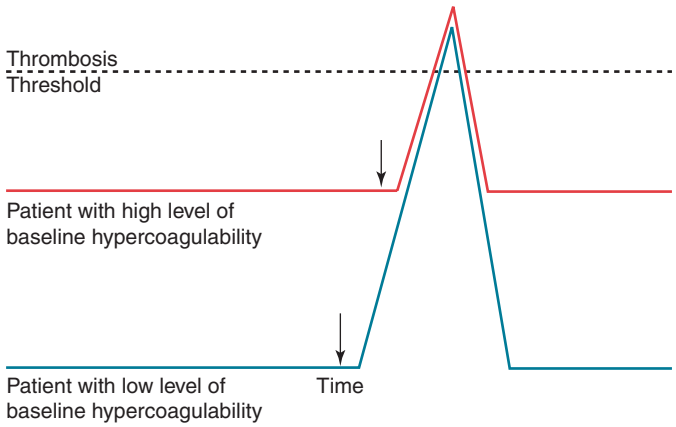
Hereditary Thrombophilia

Inherited or *hereditary thrombophilia* refers to individuals with a genetic predisposition resulting in an increased risk of thrombosis. Specific inherited alterations associated with TE have been identified (Table 35-3 ★). Most of these changes involve an increase in procoagulant potential (e.g., activation of the coagulation cascade) or a defect or decrease in natural inhibitors of clotting. Likewise, abnormalities of fibrinolysis or platelet activation can contribute to the thrombotic tendency. An acquired predisposition to thrombosis, such as prolonged immobility or pregnancy, can interact with an underlying hereditary predisposition to precipitate an acute thrombotic event. A combination of more than one hereditary predisposition strongly accelerates and exaggerates the thrombotic process. Thus, thrombosis can occur in susceptible patients having one or more genetic mutations when they are exposed to exogenous prothrombotic stimuli.[36] The "thrombotic potential" in these patients is higher than normal (Figure 35-1 ■). Inherited thrombotic disorders are usually associated with venous thrombosis.

★ **TABLE 35-3** Hereditary Conditions Associated with Thrombosis

- Antithrombin deficiency
- PC deficiency
- PS deficiency
- Activated protein C resistance (APCR)
- Prothrombin mutation G20210A
- HCII deficiency
- Tissue factor pathway inhibitor variant
- Hyperhomocysteinemia
  - Cystathionine β synthase deficiency
  - Methylenetetrahydrofolate reductase deficiency
- ABO blood type
- Dysfibrinogenemia/hyperfibrinogenemia
- Elevated FVIII
- FXII deficiency
- Plasminogen deficiency
- Plasminogen activator deficiency
- Elevated PAI-1

APCR = activated protein C resistance; HCII = heparin cofactor II; PAI-1 = plasminogen activator inhibitor; PC = protein C; PS = protein S



■ **FIGURE 35-1** Interaction of acquired thrombotic triggers with individual baseline hypercoagulability. Individuals with hereditary mutations resulting in thrombophilia have a higher baseline level of hypercoagulability (red line) compared with normal individuals (blue line). As a result, the introduction of an identical acquired prothrombotic stimulus will more easily push the thrombophilic individual into an overt acute thrombotic event (dotted line), compared with a normal individual.

Patients diagnosed with inherited thrombophilia usually present with one or more of the following clinical manifestations[37]:

1. Venous thromboembolism at a young age (before age 45)
2. Recurrent venous thromboembolism
3. Family history of venous thromboembolism
4. Thrombosis in an unusual site (cervical or visceral veins)

The five most clearly delineated hereditary abnormalities associated with thrombophilia (factor V Leiden, decreased or defective PC, PS, or antithrombin, and prothrombin 20210 mutation) combined account for ~30% of the initial cases of DVT and are the apparent hereditary defects in 75% of thrombophilic families.[38] In contrast to heterozygous female carriers of a hemophilia gene in whom 50% of normal levels of the clotting factor are sufficient to protect against bleeding, half-normal levels of anticoagulant proteins (AT, PC, PS) are associated with an increased risk of thrombosis.[39] The characteristics of these five disorders are summarized in Table 35-4 ★.[38–40]

CASE STUDY *(continued from page 721)*

Andrea's mother had had eight children and had experienced no thrombotic events although two paternal uncles had died suddenly of pulmonary embolism. Two of the mother's cousins also had pulmonary emboli: One died suddenly, but the other recovered.

1. What risk factors, if any, are revealed in the patient's history?

Antithrombin (AT) Deficiency

AT is the major inhibitor of the serine proteases involved in coagulation (thrombin, FXa, FIXa) (Chapter 32; Figure 32-1). AT's relatively weak natural inhibitory function is augmented ~1000-fold in the presence of heparin. In vivo, endothelial cell glycosaminoglycans (e.g., heparan sulfate) are the natural catalyst for AT. Reduction of AT levels to ~50% of normal predisposes to venous thrombosis because of a decrease in the normal inhibition of the coagulation pathway.

Pathophysiology

AT deficiency, the first recognized inherited risk factor for thrombosis, was described in 1965 and is inherited as an autosomal-dominant trait.[41] More than 250 mutations of the AT gene (*SERPINC1*) have been identified (www1.imperial.ac.uk/departmentofmedicine/divisions/experimentalmedicine/haematology/coag/antithrombin/).

Two major types of inherited deficiencies have been described (Table 35-5 ★). Type I deficiency is the result of reduced synthesis of a biologically normal protein (i.e., a quantitative deficiency). In this case, the antigenic and functional activities of AT are reduced in parallel. The second type of AT deficiency is produced by a specific molecular defect within the inhibitor (type II, or qualitative deficiency). Functionally, type II mutations involve either the heparin-binding site or the reactive (protease-binding) site of the AT molecule. In this case, the plasma levels of AT functional activity are reduced, whereas AT immunologic activity is essentially normal.

Clinical Findings

The prevalence of AT deficiency in the general population is about 0.02% and in patients with VTE is ~1–2%.[39] Heterozygous individuals generally have AT functional activity of 30–60% (reference interval 80–120%, or ~140 mcg [μg]/mL). Homozygous type I deficiency is not compatible with fetal survival. Homozygous type II

★ **TABLE 35-4** Inherited Thrombophilia: Characteristics of the Five Most Studied Defects

	Factor V Leiden	Prothrombin 20210	PC Deficiency	PS Deficiency	Antithrombin Deficiency
Occurrence					
General population	4–10%	2–4%	0.2–0.4%	0.07–2.3%	0.02%
First DVT	20%	7%	3%	2–3%	1–2%
Thrombophilic families	40%	18%	6%	4%	4%
Physiologic effect	FVa resistant to cleavage by APC	↑ Prothrombin ↑ Thrombin formation	↓ Inactivation of FVa, FVIIIa Unregulated coagulation	↓ Cofactor for APC ↓ APC function	↓ Inhibition of FXa, thrombin Unregulated coagulation
Clinical presentation					
Homozygous	↑↑ Risk VTE (80×)	↑ Risk VTE Possible ↑ arterial TE	Purpura fulminans	Purpura fulminans	Lethal in utero
Heterozygous	↑ Risk VTE (4–7×)	↑ Risk VTE (3–4×)	↑ Risk VTE (4–8×)	↑ Risk VTE (4–8×)	↑ Risk VTE (10×)

PC = protein C; PS = protein S; DVT = deep vein thrombosis; VTE = venous thromboembolism; APC = activated protein C; TE = thromboembolism; ↑ = slightly–moderately increased; ↑↑ = highly increased; ↓ = decreased

Adapted from: Marder VJ, Matei DE. Hereditary and acquired thrombophilic syndromes. In: Colman RW, Hirsh J, Marder VJ et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Pratice*, 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2000: 1243–275; Emmerich J, Aiach M, Morrange P-E. Thrombophilia genetics. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:962–72; Morange P-E, Tregouet D-A. Current knowledge on the genetics of incident venous thrombosis. *J Thromb Haemost*.2013;11(suppl 1):111–21.

★ **TABLE 35-5** Laboratory Classification of Congenital Antithrombin Deficiency

		Functional Activity	
	Concentration	**Heparin Cofactor**	**Progressive AT**
Type I	Decreased	Decreased	Decreased
Type II			
Active site defect	Normal	Decreased	Decreased
Heparin-binding site defect	Normal	Decreased	Normal

deficiency is usually associated with severe, life-threatening thrombotic problems in the perinatal period. Affected heterozygous individuals rarely develop thrombotic episodes before puberty, and their risk increases with advancing age. The infrequency of thrombotic events prior to puberty is thought to be due to the protective effect of α_2-macroglobulin, which is found at higher levels during the first two decades of life than in adults. About 55% of affected patients with familial AT deficiency experience thrombotic episodes (lifetime risk), most commonly involving DVT of the leg and mesenteric veins. About 60% of these individuals develop recurrent thrombotic episodes, and ~40% show clinical signs of PE.[42] Individuals with heparin-binding site defects generally have significantly less frequent and less severe thrombotic episodes than individuals with defects involving the thrombin-binding site.[43] Among the inherited thrombophilias, AT deficiency, although rare, is generally associated with the most severe clinical manifestations.[39] Single nucleotide polymorphisms (SNPs) have been reported to correlate with low-normal AT levels and increased thrombotic risk.[44–46]

Acquired deficiencies of AT also occur. Healthy newborns have about 50% of the normal adult concentration and gradually reach the adult level by 6 months of age.[43] A variety of clinical conditions—including decreased production of AT (severe hepatic disease), increased consumption of AT (acute thrombosis, disseminated intravascular coagulation [DIC]), and increased clearance or loss (urinary excretion in the nephrotic syndrome, increased clearance with heparin administration)—can reduce the concentration of AT in the blood.[47] AT levels are affected by estrogen; premenopausal women have lower levels than men. Also, AT levels decrease with high-dose (but not low-dose) estrogen OCs and in late pregnancy. The low doses of estrogen used in hormone replacement therapy generally do not affect the AT level.[38]

Laboratory Evaluation

The two major subtypes of type II AT deficiency are differentiated by two functional AT assays. The progressive AT assay quantifies the capacity of AT to neutralize the enzyme activity of a serine protease (e.g., thrombin, or FXa) in the *absence* of heparin, and the heparin cofactor assay measures heparin's ability to catalyze the neutralization of the protease by AT. Two types of AT-deficient patients have been identified using these two assays. Patients with a defect at the heparin-binding site of AT show reduced heparin cofactor activity but normal progressive AT activity. Patients with mutations affecting the thrombin-binding site have reductions in both functional assays. Immunologic assays (antigen measurements) help distinguish type I and type II defects.

Therapy

Patients with AT deficiency can usually be treated successfully with heparin for an acute thrombotic episode, although occasionally higher than usual doses of the drug are required to achieve adequate anticoagulation. If difficulty is encountered in achieving adequate heparinization or recurrent thrombosis is observed despite adequate anticoagulation, AT concentrates are available. Asymptomatic AT-deficient individuals from a thrombophilic kindred are not generally anticoagulated prophylactically unless they are exposed to situations that predispose them to developing thrombosis.[38] Patients with recurrent thrombotic disease may require lifelong antithrombotic treatment with oral anticoagulants (warfarin or Coumadin).

Protein C Deficiency

PC is a vitamin K-dependent inhibitor of coagulation (Chapter 32). PC is converted to an activated form (activated PC [APC]) by thrombin bound to endothelial cell thrombomodulin[48] (Figure 32-19). APC degrades FVa and FVIIIa in the presence of its cofactor, PS, and Ca^{++} (Figure 35-2 ■). PC in adults is present in plasma at concentrations of about 3–5 mcg/mL (reference interval 70–140%).[49] Term newborns have PC levels of ~25–45% of adult levels, which slowly rise to adult levels during adolescence.[50]

Pathophysiology

Reduction of PC levels to ~50% of normal predisposes to venous thrombosis. The diminished capacity to destroy FVa and FVIIIa results in an increased production of thrombin, which generates fibrin (a procoagulant effect). In addition, the loss of the cytoprotective effects (anti-inflammatory and anti-apoptotic) of APC binding to EPCR (endothelial protein C receptor) contribute to the thrombotic risk associated with PC deficiency.[40]

PC deficiency is inherited in an autosomal-dominant fashion. Griffin first described the hereditary deficiency of PC in 1981.[51] Similar to AT deficiency, there are two major types of congenital PC deficiency. Type I (the more common form) is characterized by a decrease of both antigen (immunologic) and functional activity to levels of 40–60% of normal (reference interval 70–140%). In type II deficiency, there are normal PC antigen levels by immunologic assay but reduced functional levels (a qualitative abnormality of the protein) (Table 35-6 ★). More than 180 different mutations have been identified for the PC gene (*PROC*) (www.itb.cnr.it/procmd/).

Recent genetic screening has revealed several SNPs in the promoter of the *PROC* gene that mildly affect plasma levels of PC.[40] In addition, a SNP resulting in a serine-to-glycine substitution at amino acid #219 of the EPCR gene (*PROCR*) affects PC plasma

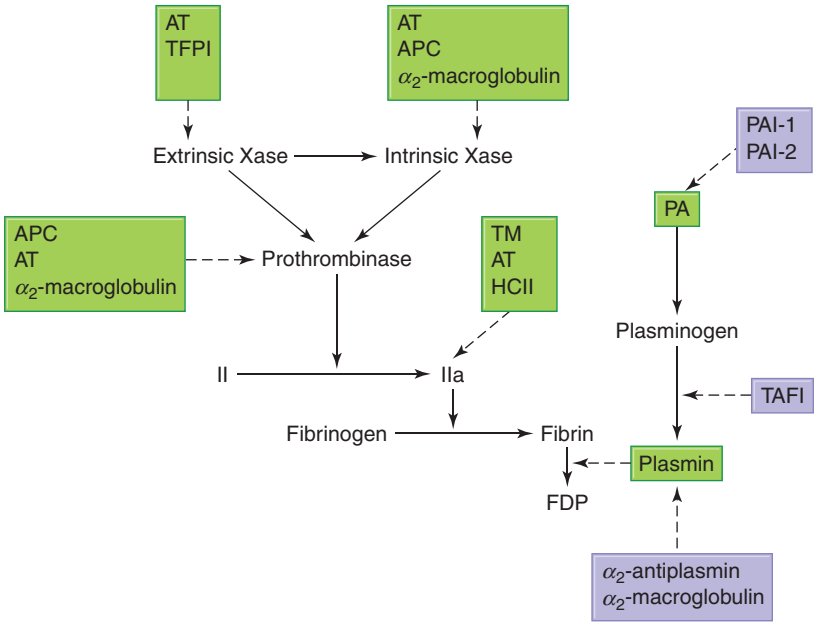


FIGURE 35-2 Physiologic control of the hemostatic process. The extrinsic Xase and intrinsic Xase activate FX, which forms the prothrombinase complex with FVa, Ca^{++}, and phospholipid. The prothrombinase complex cleaves prothrombin (II) to form thrombin (IIa). Thrombin cleaves fibrinogen to form fibrin. Plasmin cleaves fibrin and forms fibrin degradation products (FDPs). The inhibitors of each of these pathways are depicted in boxes with broken arrows to show the site of inhibition. If there is a defect or deficiency in any of the inhibitors, the hemostatic control mechanism is tipped in favor of thrombosis.

AT = antithrombin; TFPI = tissue factor pathway inhibitor, APC = activated protein C; TM = thrombomodulin; HCII = heparin cofactor II; TAFI = thrombin-activatable fibrinolysis inhibitor; PA = plasminogen activator; PAI = plasminogen activator inhibitor

levels and has been shown to be associated with a mild increase risk of VT.[52]

Clinical Findings

Heterozygous PC deficiency is found in ~0.2% of the general population and accounts for about 6% of families with thrombophilia.[38] PC-deficient individuals who develop thrombosis typically present with DVT of the legs and mesenteric veins. About 63% of affected patients develop recurrent venous thrombosis, and ~40% exhibit signs of PE.[43] However, many PC-deficient individuals never experience a thrombotic episode unless presented with an additional thrombotic risk factor, either inherited or acquired. Less than 50% of heterozygous individuals experience a thrombotic event by the age of 50 years.[40] Homozygous and double heterozygous individuals with PC deficiency can present with aggressive thrombotic complications at birth evident by purpura fulminans (large bruises that become necrotic, histologically similar to warfarin-induced skin necrosis; see next paragraph), massive venous thrombosis, and intravascular coagulation.[49] Usually purpura fulminans is a complication only in patients with <5% PC activity.[40] These individuals require replacement of PC and lifetime antithrombotic treatment, usually with oral anticoagulants (warfarin). Liver transplantation has been reported to produce a definitive cure.[53] Acquired PC deficiency is seen in liver disease, severe infection and septic shock, DIC, adult respiratory distress syndrome, and the postoperative state. Oral anticoagulation and vitamin K deficiency also lead to reduced functional PC levels.[43]

Some individuals with heterozygous PC deficiency can experience skin necrosis when treated with warfarin anticoagulants, usually in the first few days of treatment.[54] This warfarin-induced skin necrosis occurs because of a transient hypercoagulable state, resulting in the formation of fibrin thrombi in the cutaneous vasculature, hemorrhage, and necrosis. With warfarin administration, all vitamin K-dependent clotting factors decrease at a rate that depends on the individual factor's half-life. PC and FVII (with the shortest half-lives) decrease within one day with levels of factors II, IX, and X falling more slowly over 2–3 days. Individuals with heterozygous PC deficiency who start with PC levels at ~50% quickly develop a transient hypercoagulable state as their levels of PC fall precipitously. The decrease

★ **TABLE 35-6** Laboratory Classification of Protein C Deficiency

	Antigen	Functional Activity
Type I	Decreased	Decreased
Type II	Normal	Decreased

of anticoagulant PC, occurring more rapidly than decreases of most of the procoagulant factors, initially disrupts the delicate hemostatic balance and precipitates a thrombotic event in the microvasculature of the skin with purpura fulminans symptoms. If this occurs, oral anticoagulation should be stopped immediately, and PC replacement therapy initiated.

Laboratory Evaluation

Various immunologic and functional techniques have been developed to measure PC levels in plasma samples (Chapter 36). Some patients with type II deficiency have normal antigen levels and reduced functional levels in a clotting assay but normal or nearly normal amidolytic activity in chromogenic assays. Therefore, clotting assays are better functional assays for screening for PC deficiency.[39] Testing should not be done while the patient is on oral anticoagulant (Coumadin) therapy.

Therapy

Initial treatment for thrombosis associated with PC deficiency is the same as that for most other thrombotic events: heparin followed by oral anticoagulation. Because many heterozygous PC deficient individuals never experience a thrombotic episode, prophylactic oral anticoagulation may not be needed unless a secondary risk factor is present.[49] PC concentrates are available but usually necessary only to treat purpura fulminans in newborns.

Protein S Deficiency

PS is a vitamin K-dependent protein that functions as a cofactor for APC inactivation of FVa and FVIIIa (Chapter 32; Figure 32-19). The plasma concentration in normal adults is ~23 mcg/mL (reference interval 65–140%).

Pathophysiology

PS circulates in two forms: an inactive form bound to C4b-binding protein (C4BP) (~60% of total PS) and an unbound or free form (~40% of total PS). Only free PS has PC cofactor activity. The absolute and relative amounts of PS that are free are influenced by the plasma concentration of C4BP. Although C4BP is an acute-phase reactant, during an acute-phase response, only the α-chains of C4bBP are increased, and the amount of free PS is unaffected.[55] Decreases of free PS contribute to a prothrombotic tendency because of inadequate APC inactivation of FVa and FVIIIa. In addition, PS interacts directly with components of the prothrombinase complex (FVa and FXa) reducing the ability of FXa to protect FVa from degradation by APC. Loss of this function in PS deficiency also contributes to the sustained levels of FVa and increased thrombotic risk.[55]

First described and linked to a thrombotic tendency in 1984,[56] PS deficiency is inherited in an autosomal-dominant manner. The three types of congenital PS deficiency are defined on the basis of total and free PS antigen and APC cofactor activity (Table 35-7 ★). Type I PS deficiency is characterized by low total antigen, free antigen, and functional activity. Type II deficiency is characterized by normal concentration of both total and free antigen but decreased functional activity (dysfunctional molecule). Type III deficiency is characterized by normal total PS but a decreased free and functional PS concentration (reflecting either a high C4BP concentration in plasma or an abnormal binding of PS to this carrier protein). More than 200 mutations causing PS deficiency have been described (www.hgmd.org).

Clinical Findings

The prevalence of PS deficiency in the general population is ~0.7–2.3%, and it accounts for ~6% of cases of familial thrombophilia.[39] The clinical presentation of heterozygous PS deficiency is similar to that of PC deficiency, although PS deficiency has been implicated in some cases of arterial thrombosis.[38] Like PC, PS has been linked with warfarin-induced skin necrosis and in neonatal purpura fulminans when levels approach 0%. Total PS antigen level in healthy full-term newborns is 15–30% of that in normal adults; however, C4BP is also markedly reduced (20% of adult levels); thus free PS/functional levels are only slightly reduced compared with those of adults.[57] Acquired PS deficiencies are seen during pregnancy, in association with the use of OC and hormone replacement therapy, in acute TE disease and DIC, in liver disease, in cases of acute bacterial or viral disease (inflammatory states), in vitamin K deficiency, and during oral anticoagulant therapy.[38]

Laboratory Evaluation

Laboratory evaluation of PS should include assays of both total and free PS (generally immunologic assays) and a functional assay based on the ability of PS to serve as a cofactor for the anticoagulant effect of APC. Plasma level of PS in normal adults is ~23 mcg/mL. There is considerable overlap of PS levels in heterozygous PS-deficiency patients and the low-normal reference interval; thus, diagnosis may require the performance of multiple assays to establish the diagnosis of PS deficiency.[39] Activated PC resistance (see the section "Activated Protein C Resistance") was reported to cause false positive test results when screening for PS deficiency if clotting (functional) assays are used. However, assays using FV-deficient plasma as the substrate have improved the specificity of PS assays.[58]

Therapy

Treatment for PS deficiency is similar to that for PC deficiency. PS concentrates have not yet been developed for clinical use. Coumadin-induced skin necrosis is a rare complication of oral anticoagulation in PS deficiency.

★ **TABLE 35-7** Laboratory Classification of Protein S Deficiency

	Total Protein S	Free Protein S	Protein S Activity
Type I	Decreased	Decreased	Decreased
Type II	Normal	Normal	Decreased
Type III	Normal	Decreased	Decreased

☑ CHECKPOINT 35-2

Why are both clotting and immunologic assays for PS, PC, and AT necessary when evaluating an individual for inherited thrombophilia associated with these proteins?

Activated Protein C Resistance

Thrombin activates PC to APC, which in turn inhibits coagulation by degrading FVa and FVIIIa. A novel mechanism for familial thrombophilia was recognized in 1993 with the description of a syndrome characterized by inherited resistance to APC, **activated protein C resistance (APCR)**.[59]

Pathophysiology

APCR is characterized by APC's inability to prolong clotting tests when it is added to the test system. The addition of APC to plasma normally results in a prolongation of the clotting time because of the destruction of FVa and FVIIIa. Individuals with APCR exhibit a diminished ability of APC to destroy FVa. In vivo, inadequate FVa inactivation can lead to increased production of thrombin and possibly thrombosis. About 90% of APCR cases are the result of a single-point mutation of the FV gene (*F5*), involving replacement of Arg 506 with Gln (FV^{R506Q}, also called **factor V Leiden [FVL]**). This makes the mutant FVa molecule resistant to APC inactivation by altering an APC cleavage site.[60] In addition, FV serves as a cofactor in the APC inactivation of FVIIIa. FVL is a much less effective cofactor in this inactivation of FVIIIa, which contributes to the thrombophilic state.[61]

Studies of the 10% of individuals with APCR who do not have the FV^{R506Q} mutation are ongoing. A mutation at Arg 306 (a second APC cleavage site in FVa) has been reported that also demonstrates low APC sensitivity.[62–64] However, this mutation does not seem to be linked to a thrombotic tendency.[65] Two mutations in FV that do not affect APC cleavage sites (FV Ile359Thr and FV Glu666Asp) but *are* associated with both APC resistance and an increased risk for thrombosis have recently been identified.[66] So far, no genetic abnormalities in the FVIII gene have been identified as causing APC resistance in humans.[67]

Clinical Findings

The prevalence of heterozygous FVL mutation in Caucasians ranges from 2–15%, depending on the geographic population studied.[39] The mutation is rare in Africans, Asians, Australians, and Native Americans.[68] FVL is seen in as many as 10–25% of patients with venous thrombosis and accounts for about 40% of the cases of familial thrombophilia.[38] Homozygotes are at higher thrombotic risk than heterozygotes. Heterozygotes with FVL are unlikely to develop thromboses unless other hereditary (e.g., mutations in PC, PS, AT)[25] or acquired risk factors are present (OC, pregnancy, trauma). Acquired APC resistance (not related to FVL) has been reported during pregnancy, with the use of OC, and in patients with lupus anticoagulant.[39]

Laboratory Evaluation

Laboratory tests for APCR are either clot-based functional tests or molecular techniques. The functional test evaluates the prolongation of the APTT after the addition of purified APC to the test system. Results are expressed as an APC sensitivity ratio: APTT (+APC)/APTT(−APC). A low APC sensitivity ratio defines APCR.[39] The confirmatory test for FVL is a PCR-based molecular assay. Polymerize chain reaction (PCR)–based confirmatory tests for APCR resulting from mutations other than FVL are available in research laboratories only. Routine clinical diagnosis requires both clotting (screening) tests for APCR and molecular (PCR) tests for FVL because 10% of individuals with APCR do not have the FVL mutation.

Therapy

The management of acute thrombosis in patients with APCR is the same as in other thrombotic patients. Individuals with heterozygous APCR are usually given prophylactic antithrombotic treatment only if they are in a situation of increased thrombotic risk or have recurrent thrombosis.

Prothrombin Gene Mutation 20210

Prothrombin is cleaved by the activated FXa–FVa complex to form thrombin.

Pathophysiology

In 1996, it was reported that a G ⟶ A substitution in the 3′ untranslated region (nucleotide 20210) of the prothrombin gene (*F2*) is associated with a mild elevation of plasma prothrombin levels (115–130% of normal) and an increased risk of venous thrombosis.[69] The prothrombin mutation (G20210A) is the most recent of the hereditary thrombophilias to have a clear-cut genetic mutation defined. The elevated plasma prothrombin level can directly contribute to an increased thrombotic risk by causing increased thrombin generation. In addition, decreased fibrinolytic activity can occur because of enhanced activation of TAFI. A second SNP in the *F2* gene (A19911G) that is also associated with elevated FII levels has been reported, but its association with an increased risk of thrombosis is unclear.[70]

Clinical Findings

The prevalence of this autosomal-dominant gene is ~1–2% among Caucasians, and like FVL, it is infrequent among persons of African, Asian, and Native American origin. It is estimated that ~6% of thrombotic patients are heterozygous for this mutation and that it accounts for about 18% of cases of familial thrombophilia (making it the second most common cause after FVL mutation).[22] A substantial percentage of symptomatic individuals with the prothrombin mutation also had the FVL mutation or another genetic risk factor for thrombosis,[71] supporting the concept that thrombophilia is a multirisk factor disease. Individuals homozygous for the prothrombin mutation have a significantly higher risk of VTE and can have an increased risk of arterial thrombosis.

Laboratory Evaluation

Screening for this mutation using coagulation testing is unreliable because prothrombin levels in these patients overlap with values in the normal population. Molecular testing (PCR) for the single point mutation at position 20210 in the prothrombin gene is confirmatory.

Therapy

Patients with this disorder are treated the same way as are other thrombotic patients. Prophylatic treatment can be given in high-risk situations.

☑ CHECKPOINT 35-3

A patient was being evaluated for inherited thrombophilia. The results for the clotting assay for activated PC resistance were positive, but the FVL molecular assay was negative. Are these results inconsistent?

Other Potential Genetic Risk Factors

Heparin Cofactor II Deficiency

HCII is a second thrombin inhibitor, and like AT, its action is accelerated by heparin, and also by dermatan sulfate. In contrast to AT, which inhibits most of the procoagulant serine proteases, HCII inhibits primarily thrombin. HCII deficiency is inherited as an autosomal-dominant disorder, and although low levels are associated with thrombosis, it does not appear to be a strong risk factor.[39] Many individuals with this defect are asymptomatic, and a partial deficiency (heterozygous state) is likely a thrombotic risk factor only if it is combined with another thrombophilic risk factor (inherited or acquired).[72,73] Acquired HCII deficiency can be seen in patients with liver disease and in DIC. Several assays are available for measuring HCII, but none is currently Food and Drug Administration (FDA) approved and thus not available in routine coagulation laboratories. Because both HCII and AT inhibit thrombin, the recommendation is that functional assays for AT be based on FXa inhibition rather than thrombin inhibition to improve assay specificity for AT.

Tissue Factor Pathway Inhibitor Variant

Tissue factor pathway inhibitor (TFPI) is a natural inhibitor of coagulation that directly neutralizes FXa and, in complex with FXa, neutralizes the tissue factor:VIIa complex.[74] TFPI exists in vivo primarily bound to endothelial cells; thus, measurement of plasma levels (representing ~10–15% of total TFPI) may be uninformative in identifying a deficiency state that could predispose to thrombophilia.[75] A weak association between low levels of plasma TFPI (≤10th percentile) and thrombotic disease has been reported.[76] In addition, a candidate mutation in the *TFPI* gene resulting in a proline to leucine substitution at position 151 of TFPI has been reported to have an increased risk of VTE.[77]

ABO Blood Group

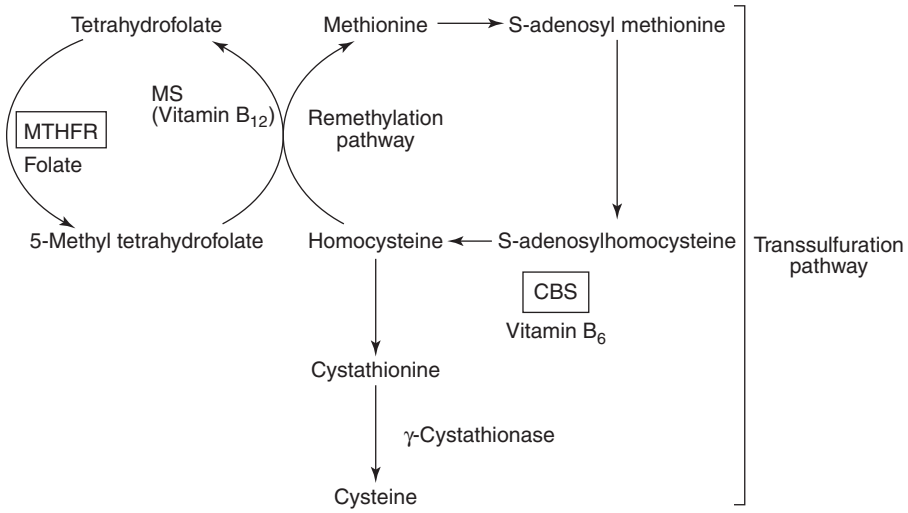
Non-O blood groups have been reported to be associated with an increased risk of VT with B and A1 blood groups at higher risk than O and A2 blood groups.[78,79] Much of this effect is thought to result from the effect of the ABO locus glycosyltransferases on von Willebrand factor and FVIII, resulting in a higher level of both proteins in non-O blood groups.

Hyperhomocysteinemia (HC)

Homocysteine (HC) is an amino acid that is metabolized either by transsulfuration to cystathionine or by remethylation to methionine (Figure 35-3 ■). The two main enzymes involved in HC metabolism are cystathionine β synthase (CBS) and methylene tetrahydrofolate reductase (MTHFR). Normal plasma levels of HC are ~5–15 mcmol/L, and HC levels increase with age.

Pathophysiology

Hyperhomocysteinemia (plasma homocysteine level above the reference interval) results when homocysteine metabolism is impaired. Severe hyperhomocysteinemia (plasma HC >100 mcmol//L) is associated with homocystinuria and is seen in a group of rare inborn errors of metabolism. The most common causes for homocystinuria are homozygous mutations in the CBS gene; mutations less commonly



■ **FIGURE 35-3** Homocysteine metabolism. Mutations of MTHFR and CBS are associated with hyperhomocysteinemia. Deficiencies of vitamin B_{12}, vitamin B_6, and folate are associated with acquired hyperhomocysteinemia. Hyperhomocysteinemia can lead to increased risk for thrombosis.

MTHFR = methylene tetrahydrofolate reductase; MS = methionine synthetase; CBS = cystathionine ß-synthase

occur in the MTHFR gene. Homocystinuria is associated with premature atherosclerosis (cardiovascular disease and stroke) and venous and arterial thrombosis.[80] Subsequently, less severe elevations of blood homocysteine levels were implicated in VTE.[81]

The exact mechanisms that link homocysteine to thrombosis are not fully understood, but the major effect is on the vessel wall. Mild to moderate homocysteinemia is now recognized to be an independent risk factor for both arterial and venous thrombotic disease. Up to 40% of patients with atherothrombotic coronary or cerebrovascular disease have elevated plasma homocysteine as do 13–18% of patients with an initial episode of VTE before age 45.[43]

Clinical Findings

Both inherited and acquired conditions can cause increased HC levels. Multiple genetic mutations of CBS and MTHFR have been identified. Although homozygous CBS deficiency is the most common cause of homocystin*uria*, the most common genetic causes of mild homocystein*emia* involve mutations of the *MTHFR* gene. A *MTHFR* gene polymorphism (nucleotide C677T ⟶ Val replaces Ala222) is the *MTHFR* mutation believed to be responsible for the majority of cases of a hereditary predisposition to mild hyperhomocysteinemia.[82] Thirty to 40% of the Caucasian population are heterozygous for this polymorphism but typically do not experience hyperhomocysteinemia or increased risk for vascular disease without other thrombotic risk factors. The most common causes of acquired hyperhomocysteinemia are deficiencies of vitamin B_{12}, folate, or vitamin B_6 (which are cofactors in HC metabolism), smoking, some medications, aging, hypothyroidism, diabetes, and renal disease.[81]

Laboratory Evaluation

HC can be measured by gas chromatography, ion exchange chromatography, and high-performance liquid chromatography. The C677T *MTHFR* mutation can be identified using molecular assays. MTHFR heterozygotes or individuals with inadequate vitamin B_6 levels can have normal or only slightly elevated levels of HC. Significant age and gender differences in HC levels exist.

Therapy

Treatment of hyperhomocysteinemia usually involves dietary manipulation or vitamin therapy. Administration of vitamin B_6, vitamin B_{12}, or folate often results in normalization of homocysteine levels, but no clinical efficacy of this approach has been demonstrated in controlled clinical trials.[81]

Fibrinogen Disorders

Fibrinogen is cleaved by thrombin to form fibrin. Fibrinogen can be qualitatively or quantitatively abnormal. Qualitative abnormalities of fibrinogen usually show autosomal-dominant inheritance patterns.

Pathophysiology

Dysfibrinogenemias are a heterogeneous group of disorders that result in a structurally altered fibrinogen molecule that can lead to altered fibrinogen function. More than 600 different mutations have been identified as causing dysfibrinogenemias.[83]

Dysfibrinogenemias can present with no clinical symptoms (~55% of cases), a bleeding diathesis (~25% of cases), or a history of recurrent venous or arterial thromboembolism (~20% of cases).[84] Alterations of fibrinogen predisposing to bleeding include impaired release of fibrinopeptides, defective fibrin polymerization, and abnormal cross-linking by FXIIIa. Two types of thrombotic dysfibrinogenemias appear to be associated with decreased fibrinolytic activity in vivo (thus predisposing to thrombosis): one is abnormally resistant to lysis by plasmin (mutations of plasmin cleavage sites in fibrin), and the other is associated with reduced plasminogen activation (mutations of binding sites for plasminogen or plasminogen activators). In addition, fibrinogen mutations that result in fibrin clots with a high fiber density also retard fibrinolysis.[85] A mutation in the fibrinogen gamma chain gene (*FGG*) (C to T mutation at position 10034) has been associated with an increased risk of VT.[85]

Interestingly, about 30% of individuals with fibrinogen deficiencies (hypofibrinogenemia) actually present with thrombosis. One possible explanation for this paradoxical observation is the stability of the fibrin clot formed. Less dense, more fragile or unstable fibrin clots may be more likely to break apart and embolize, resulting in thrombi in downstream microvascular beds.[86]

Clinical Findings

Patients with thrombotic dysfibrinogenemias are reported to have problems related to pregnancy (spontaneous abortions, postpartum thromboembolism).[87]

Laboratory Evaluation

Both quantitative (immunologic assays) and functional tests (thrombin times, reptilase times) for fibrinogen should be performed when dysfibrinogenemia is suspected. Functional fibrinogen measurements are usually significantly lower than antigenic measurements in the plasmas of individuals with dysfibrinogenemia.

Elevated Factor VIII and VWF

Individuals with increased plasma levels of FVIII and/or VWF (>150% of normal) have an increased risk of thrombotic disease, possibly because of enhanced thrombin formation or diminished anticoagulant effect of APC.[88] A dose–response relationship seems to exist between FVIII level and VTE risk.[89] FVIII levels increase with aging, pregnancy, surgery, chronic inflammation, liver disease, and exercise. Specific molecular polymorphisms of the *F8* and *VWF* genes recently have been associated with elevated levels of the respective proteins.[90,91] Interestingly, a genome-wide association study identified six gene loci other than *VWF* that modulated VWF plasma levels, three of which also influenced FVIII levels. Of the six, three have been shown to be associated with increased VT risk.[92]

Elevated Levels of Other Coagulation Factors

The Northwick Park Study was one of the first to evaluate hemostatic measurements as possible thrombogenic risk factors.[93] This study showed a strong positive association between thrombosis and increased levels of FVIIa and fibrinogen. The relative risk for cardiovascular disease is nearly three times higher for individuals in the highest quintile of fibrinogen concentration compared with those in the lowest. Increased levels of other factors have been reported to be linked with an increased risk for venous thrombosis.[94] These include elevated FIX (>129% of normal)[95] and FXI (>121% of normal).[96] Rare variants of the *F2* and *F9* genes were recently shown to be associated with increased risk of VT.[97,98]

Factor XII and Thromboembolic Disease

Patients with FXII deficiency have a prolonged APTT but no clinical bleeding phenotype. VTE, myocardial infarction, and stroke have occurred in FXII-deficient patients and were postulated to be related to the role of FXII in activating fibrinolysis. However, most of these patients had additional FXII-independent risk factors for VTE, which were more likely related to the thrombosis. FXII deficiency has been suggested to possibly contribute to secondary embolic disease because of defective thrombus stability.[99] Recent studies linking the contact system with pathologic thrombus formation have suggested that deficiency of FXII (and other proteins of the contact system including FXI) may provide thromboprotection.

Fibrinolytic System Disorders

Hereditary disorders of fibrinolysis are less frequently associated with thrombotic disease than are hereditary anomalies of procoagulants or coagulation inhibitors.[100] Possible causes of abnormalities of fibrinolysis include the structure of fibrin (see the section "Fibrinogen Disorders"), the amount and function of plasminogen, and the physiology of plasminogen activation (impaired plasminogen activator synthesis or release, increased plasminogen activator inhibitor, or increased levels of TAFI). Whatever the cause, an inadequate fibrinolytic response would delay lysis of the thrombus and/or contribute to its expansion.

Plasminogen Deficiency

Plasminogen abnormalities have been classified as either type I (decrease of both protein concentration and functional activity) or type II (normal antigen levels but reduced functional activity [dysplasminogenemia]). Multiple genetic polymorphisms of the plasminogen gene (*PLG*), including silent (null) alleles, have been described.[101] However, the association of the heterozogous state for a mutant *PLG* allele as a predisposing risk factor in VTE is not firmly established. Laboratory tests are available to measure protein levels (immunologic) and functional activity (clot-lysis or chromogenic substrate) of plasminogen.

Tissue Plasminogen Activator (tPA) and Plasminogen Activator Inhibitor (PAI)

Inadequate stores or release of tPA or an inappropriate/excess release of PAI could theoretically result in thrombotic events (Figure 35-1). Several reports have linked defective fibrinolysis associated with decreased release of tPA and/or elevated PAI-1 with familial thrombosis. However, two of these families subsequently were shown to have hereditary PS deficiency, the more likely cause of clinical disease in these families.[102,103] tPA and PAI-1 are thought to likely play a greater role in acquired thrombophilia than in hereditary thrombophilia. Functional (chromogenic assays) and quantitative (enzyme-linked immunosorbant assay [ELISA]) assays for tPA and PAI-1 are available.

☑ CHECKPOINT 35-4

Why is thrombotic disease associated with hereditary thrombophilia considered a multigene (or multirisk factor) disease?

CASE STUDY (continued from page 726)

Andrea was initially tested while she had deep vein thrombosis and again six months later after being taken off Coumadin. The tests included AT, PC, PS, fibrinogen, D-dimer, and plasminogen. All test results were in the reference range on both occasions.

2. What other possibilities could explain the thrombotic event in this patient?

Acquired Thrombohemorrhagic Conditions

The acquired or secondary thrombophilic states include a heterogeneous group of disorders in which an increased risk appears to exist for developing thromboembolic complications. The pathophysiologic basis for the thrombophilic state in most of these situations is complex. Various clinical conditions are associated with a high risk of thrombotic complications (Table 35-8 ★). As with the hereditary thrombophilias, acquired disorders vary widely in their tendency to cause

★ **TABLE 35-8** Secondary Disorders and Other Factors Contributing to Acquired Thrombosis

Secondary disorders

- Fibrinolytic system defects
- Antiphospholipid antibody syndrome
- Malignancy
- Hip, knee surgery
- Hematologic disorders
  - Polycythemia vera/myeloproliferative disorders
  - Hemolytic anemia
  - Sickle cell anemia
  - Paroxysmal nocturnal hemoglobinuria
  - Acute promyelocytic leukemia
- Chronic inflammatory disease
- Congestive heart failure
- Atrial fibrillation
- Nephrotic syndrome
- Atherosclerosis
- Hyperlipidemia, hypercholesterolemia, increased lipoprotein (a)

Other factors

- >50 years of age
- Obesity
- Diet with inadequate folate, vitamin B_6, or vitamin B_{12}; chronic intake of fatty food
- Smoking
- Pregnancy, use of oral contraceptives, hormone replacement therapy
- Stasis/immobilization (prolonged car or air travel; bed rest)
- Trauma/postoperative state

venous and arterial thrombotic disease (Table 35-3). Acquired defects leading to thrombosis are at least as common as inherited deficiencies and often precipitate the acute thrombotic episode in the patient with an inherited thrombophilia.[2] In a number of acquired conditions associated with a thrombophilic tendency, the clinical presentation can range from microvascular and macrovascular thrombosis to bleeding (hence the term *thrombohemorrhagic conditions*).

Acquired Fibrinolytic Defects

Early studies of fibrinolytic function in patients with thromboembolic disease consistently demonstrated that 30–40% of these patients had an impaired fibrinolytic function.[104] Decreased fibrinolysis has been linked to many acquired conditions including postsurgery, coronary disease, taking OCs, the last trimester of pregnancy, certain infections, aging, radiation therapy, some drugs, and malignancy.[105] Increased plasma PAI-1 level is the most common reason for an impaired fibrinolytic function although this increase is often combined with a decreased capacity to release tPA.[104] Many patients with DVT have a history of a long-lasting inflammatory response, which contributes to an impaired fibrinolytic function by elevating the PAI-1 levels (an acute-phase reactant). An increased PAI-1 level in plasma can predict the development of a new thrombotic event (postoperatively, in myocardial infarction, and idiopathic DVT).[104]

Antiphospholipid (aPL) Antibodies/Antiphospholipid Syndrome (APS)

The **antiphospholipid syndrome (APS)** has multiple clinical manifestations and is the most common cause of acquired thrombophilia.[37] **Antiphospholipid antibodies (aPLs)** include a broad group of autoantibodies that includes the lupus anticoagulant (LA), anticardiolipin antibodies (aCL), and several subgroups (antibodies that recognize other phospholipids and phospholipid-binding proteins).[106] These antibodies prolong phospholipid-dependent clotting assays in vitro (Chapter 34).

Phospholipid-reactive antibodies (aPLs) were first described in patients with biologic false-positive serologic tests for syphilis and were shown to recognize cardiolipin within the test reagent (i.e., aCL).[107] The same year, aPLs were identified in patients with systemic lupus erythematosus (SLE), subsequently named LA.[108] Other antibodies that reacted with anionic phospholipids other than cardiolipin (e.g., antiphosphatidylserine) were later identified. Before 1990, it was thought that aPL antibodies recognized epitopes within the anionic phospholipids, but it was subsequently demonstrated that many of them recognize protein cofactors or antigenic targets in complex with the phospholipids.[106] These aPL antibodies can be produced after certain infections (mycobacteria, malaria, other parasitic organisms), after exposure to certain medications (neuroleptics, chlorpromazine, quinidine, procainamide), and by patients with other autoimmune disorders (SLE, Sjögren syndrome, rheumatoid arthritis, immune thrombocytopenia).

Pathophysiology

Paradoxically, although these antibodies prolong in vitro coagulation assays (suggestive of defective hemostasis), individuals with aPL antibodies do not suffer from a bleeding diathesis unless other hemostatic defects are present (e.g., thrombocytopenia, hypoprothrombinemia). Rather, the presence of aPL apparently increases the risk of both arterial and venous thrombosis with approximately one-third of patients with such inhibitors having thrombotic events.[11]

The most commonly identified antiphospholipid antibodies, LA and aCL, behave similarly. Immunologically, both LA and aCL are usually IgG but can be IgM or a mixture of the two. The antibody combines with the phospholipid surfaces of test reagents used in the APTT, prolonging the clotting times. Both LA and aCL were originally thought to react only with phospholipid, but further studies revealed that they require a protein cofactor to be associated with the phospholipid. The protein targets recognized by aPL include β_2-glycoprotein-1 (β_2GP1), prothrombin, FV, FVII, PC, PS, TFPI, and heparin.

Various mechanisms have been proposed to explain the increased risk of thrombosis with aPL. APS may actually be composed of subcategories defined by the specificities of the particular antibodies.[106] Pathophysiologic mechanisms include injury and activation of endothelial cells and monocytes (causing cells in the blood or in contact with the blood to acquire a procoagulant phenotype); platelet activation; and inhibition of endogenous anticoagulant and fibrinolytic mechanisms. Specific aPL-induced changes include interference with PC activation or activity (decrease in thrombomodulin expression, decrease in free PS), inhibition of heparin sulfate interaction with AT, inhibition of endothelial cell production or release of prostacyclin, alterations in fibrinolytic mechanisms (decreased tPA and/or increased PAI-1, inhibition of TFPI). In addition, aPLs can stimulate platelet aggregation and promote tissue factor synthesis by leukocytes.

Clinical Findings

The clinical manifestations that are most frequent in these patients include systemic vascular thrombosis (DVT, PE), thrombosis at unusual sites, increased risk of arterial thrombosis (stroke, other neurological complications), and increased risk of adverse pregnancy outcomes (recurrent miscarriages). It is estimated that 5–30% of patients with VTE are positive for LA (aPL) in comparison to 3–10% of the general population.[106] *Secondary APS* describes the occurrence of aPL and thrombosis in conjunction with an autoimmune condition such as SLE. When aPL occurs as an independent autoimmune disorder, it is referred to as *primary APS*.

Laboratory Evaluation

No single test identifies every LA/aPL; instead, a combination of tests must be performed. Two types of assays are used to detect aPL antibodies: (1) coagulation assays, in which "LAs" are detected by their ability to prolong phospholipid-dependent coagulation reactions and (2) specific immunoassays, usually ELISA, that are used to detect antibodies reactive with cardiolipin, phosphatidyl serine, β_2GP1, prothrombin, or FVII. Table 34-11 in Chapter 34 shows typical laboratory test results and comparisons to other acquired conditions associated with a prolonged APTT. The sensitivity of the APTT reagent for LA detection varies with different commercial reagents used for the test. Some commercial APTT reagents have been created to be very sensitive to the presence of LA; others are marketed as being insensitive to LA.

The Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis has proposed

criteria to standardize the diagnosis of LA. It requires a four-step approach:

1. Prolongation of a phospholipid-dependent coagulation assay (usually the APTT)
2. Evidence of inhibitor activity in the patient plasma (lack of correction by a 1:1 mix with normal plasma).
3. Evidence of the phospholipid dependence of the inhibitor effect
4. Absence of a specific inhibitor against a coagulation factor

A sequence of suggested tests for evaluating a patient's plasma for the presence of LA is outlined in Table 35-9 ★.[109]

The first step in identifying LAs is that at least one laboratory test that uses phospholipid in the reagent (e.g., the APTT) must be prolonged. APTT reagents with silica as the activating agent are preferred; kaolin and ellagic acid are not recommended.[106] This prolonged test must then be repeated on a 1:1 mixture of patient's plasma and normal plasma (NP) to distinguish between an inhibitor and a single-factor deficiency (see "Mixing Studies", Chapter 36). In the presence of an LA inhibitor, no correction is noted (the LA usually prolongs the clotting time of the NP immediately after mixing in contrast to inhibitors specific for coagulation factors, which usually require incubation). A specific factor deficiency can also be ruled out by factor assays, if needed.

The possibility that the presence of heparin or other anticoagulants causes the prolonged APTT must also be eliminated. Heparin can be identified by observing a prolonged thrombin time and a normal reptilase time (Chapter 36). At least one additional abnormal screening test for the LA, such as the dilute Russell viper venom test (dRVVT), must be demonstrated.

★ **TABLE 35-9** Recommended Approach for Identifying the Lupus Anticoagulant

Screening procedures—two or more using single concentration of phospholipid

1. Demonstrate at least one prolonged phospholipid-based clotting test (e.g., APTT).
2. Demonstrate at least one additional prolonged LA screening test (e.g., dRVVT, PCT)

Confirmatory procedures—modify abnormal screening procedure by altering phospholipid content of the test procedure, which demonstrates that the LA depends on phospholipid

1. Reducing phospholipid concentration in test reagent to accentuate prolonged test (e.g., diluting the test reagent)
2. Neutralizing the effect of the LA by increasing the phospholipid in the test system (e.g., frozen-thawed platelets [platelet neutralization procedure], platelet vesicles hexagonal phase phospholipid)

Additional requirements for documenting LA

1. Mixing studies with normal platelet-free plasma not corrected
2. Exclusion of presence of heparin in the sample (e.g., normal thrombin time/reptilase time)
3. Proof that another coagulopathy is not present or concurrent; specific factor assay to rule out a factor deficiency; detailed clinical history

LA = lupus anticoagulant; APTT = activated partial thromboplastin time; dRVVT = dilute Russell's viper venom time; PCT = plasma clotting time

A confirmatory test is then performed to establish that the antibody/inhibitor is phospholipid dependent. Confirmatory tests are modifications of the screening tests originally abnormal that alter the amount of phospholipid in the test system. The modifications usually involve either reducing the amount of phospholipid in the reagent or adding an excess of phospholipid to the test system. The most sensitive tests for detecting the inhibitor contain only limited amounts of procoagulant phospholipids (dRVVT or dilute APTT). The addition of excess phospholipid to the test system causes an overabundance of phospholipid, overwhelms the antibodies, and shortens the original prolonged test time to within the normal reference interval. The *platelet neutralization procedure* (Chapter 36) is a popular confirmatory test that adds washed, frozen and thawed platelets, an abundant source of phospholipid, to the patient's plasma; the prolonged screening test is corrected. A better confirmatory test is the *hexagonal phase phospholipid test* (Chapter 36). The LA can recognize phosphatidylethanolamine in the hexagonal phase array configuration (hexagonal H_{11}) but not in the lamellar phase. Adding H_{11} phase phosphatidyl ethanolamine to an LA patient specimen corrects the prolonged APTT by absorbing the LA antibodies. Unlike the platelet neutralization procedure, the hexagonal phospholipid test does not give false positives with heparin. See Figure 35-4 ■ for an outline of a flow chart for an approach to laboratory testing for LA.

aCL IgG and IgM assays are the most sensitive but least specific assays, while anti-β_2GPI IgG and IgM assays are more specific but less sensitive. LA coagulation assays are the least sensitive, but the most specific assays in testing for APS.[106]

Therapy

Management of acute VTE in patients with APS is similar to that of other individuals with thrombosis. However, the APTT cannot be used reliably to monitor unfractionated heparin dosage without modifications (e.g., the use of an insensitive APTT reagent or the activated coagulation time/ACT test). Low-dose heparin circumvents this problem because it does not require laboratory monitoring (see the section "Heparin"). Patients who develop transient LA/aPL in association with infections do not usually experience TE episodes.[106] The presence of a persistent LA and/or a high-titer antibody in an asymptomatic patient with no prior thrombotic history is not an indication for anticoagulant therapy unless there are coexisting clinical circumstances (surgery, prolonged immobilization).

Heparin-Induced Thrombocytopenia (HIT)

Heparin is used to prevent or treat thrombosis. However, its use can cause thrombocytopenia in some patients, generally associated with platelet activation. **H**eparin-**a**ssociated **t**hrombocytopenia (HAT) in which the thrombocytopenia results from a direct, *nonimmune*-mediated platelet activation, is not associated with an increased risk of thrombosis. **Heparin-induced thrombocytopenia (HIT)** is an immune-mediated thrombocytopenia caused by an autoantibody directed against heparin complexed with platelet factor 4 (PF4).[110]

Pathophysiology

In HIT, PF4 is released from platelet α-granules during platelet activation, nonspecifically binds to heparin in the circulation, and antibodies are produced to the heparin/PF4 complex. The antibody/

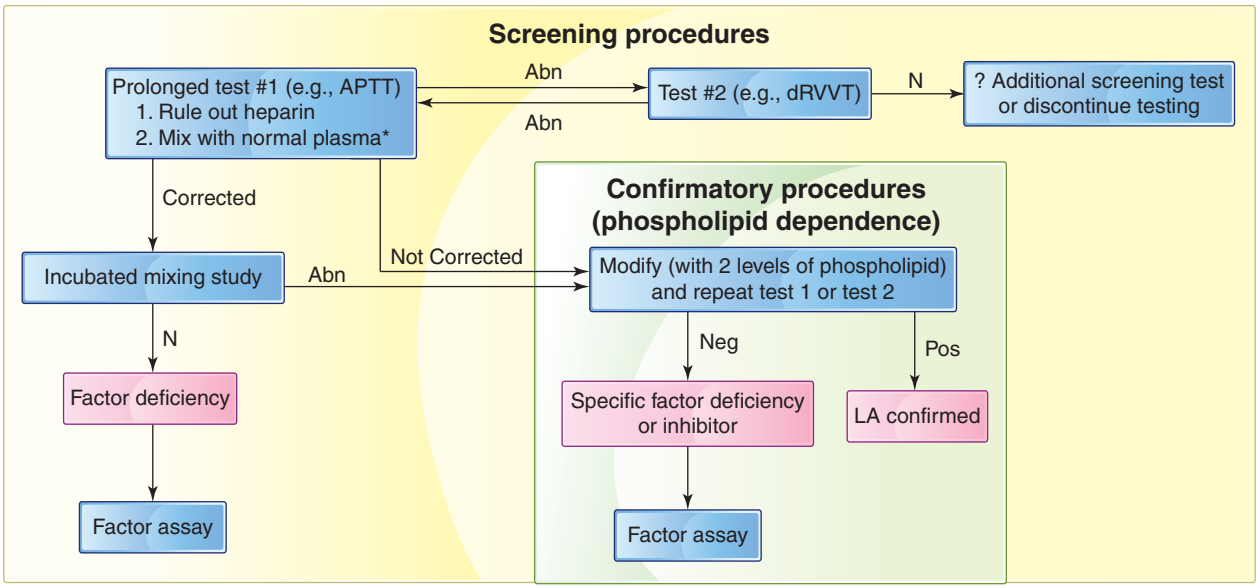


FIGURE 35-4 A flow chart for an approach to the diagnosis of the lupus anticoagulant (LA). At least two phospholipid-based screening tests (tests using one concentration of phospholipid) must be prolonged. Mixing studies must be uncorrected with normal plasma. The presence of heparin must be ruled out. Confirmatory tests are performed by modifying the abnormal screening tests with two levels of phospholipid.

*Plasma samples must be platelet free, Cor = corrected; N = normal; Abn = abnormal; LA = lupus anticoagulant; APTT = activated partial thromboplastin time; dRVVT = dilute Russell's viper venom time

heparin/PF4 complex then attaches to the platelet surface via the platelet FcγIIa receptors, resulting in increased platelet clearance and thrombocytopenia. The antibody/heparin/PF4 complexes can induce platelet activation and aggregation.

Clinical Findings

The most serious complication of HIT is activation of the coagulation system and thrombosis, which can be life threatening. *Isolated HIT* refers to the patient with thrombocytopenia without thrombosis, whereas *HITT* is used to refer to patients with HIT and thrombotic complications. A number of events are thought to contribute to the thrombotic complications including in vivo platelet activation, generation of procoagulant platelet-derived microparticles, and activation of endothelium and expression of tissue factor via HIT antibody recognition of PF4/endothelial heparan sulfate complexes.[110] Thrombocytopenia develops in approximately 3% of patients who receive **unfractionated heparin (UFH)**, and thrombosis occurs in one-third of those.[111] The most common venous thrombotic complications are DVT and PE, and arterial thrombosis most commonly affects the lower limb (acute limb ischemia) or cerebral vessels (thrombotic stroke). **Low-molecular-weight heparin (LMWH)** can trigger HIT but much less frequently than UFH.[112] Fondaparinux, a synthetic heparin pentasaccharide, has the lowest risk of inducing HIT (<0.01%).[112]

HIT is suspected when a patient receiving heparin demonstrates a thrombocytopenia of $<150 \times 10^9$/L or a 50% drop in platelet count from baseline. Typically, the onset is 5–14 days after starting any dose, any type, or any route of heparin exposure.[112] A more rapid fall (within hours) in platelet count occasionally occurs if the patient has been treated with heparin within the previous 100 days. Because heparin is generally used in patients who have experienced an acute thrombotic episode or who are at risk of one, other causes of thrombocytopenia must be excluded.

Laboratory Evaluation

Two types of assays are available to diagnose HIT: functional and antigen. Functional assays based on the ability of HIT-IgG to activate platelets, either the platelet serotonin release assay (SRA) or heparin-induced platelet activation assay (HIPA), are most commonly used.[112] ELISA assays for HIT-IgG have been developed using the heparin/PF4 target antigen. Both types of assays have similar sensitivity for HIT, but activation assays have greater diagnostic specificity. Only platelet-activating IgG antibodies are capable of causing HITT.[112] Most routine hospital laboratories do not offer functional assays for HIT, but they may be available through reference labs. The HIT antibodies usually remain detectable for only 4–6 weeks. Routine screening for HIT antibodies in patients who are receiving heparin is not recommended because many patients can form HIT antibodies without developing thrombocytopenia or experiencing other adverse events.[112]

Therapy

Patients receiving UFH should have their platelet counts monitored periodically during therapy, beginning on day 5 of heparin use. If the platelet count falls, heparin should be discontinued and another form of anticoagulant therapy begun (e.g., the heparinoid danaparoid or a direct thrombin inhibitor such as hirudin or argatroban). Although LMWH is a less frequent inducer of HIT, once the antibodies have formed, LMWH cannot be used because it also binds PF4 and the HIT antibody. Oral anticoagulants should not be started until adequate anticoagulation with these alternative agents has been achieved and the platelet count has returned to the normal range.

☑ CHECKPOINT 35-5

A patient was being evaluated for HIT. The ELISA assay for HIT IgG was positive, and the platelet SRA was negative. Should this patient's physician be concerned? Why or why not?

Thrombotic Microangiopathies (TMA)

The term *thrombotic microangiopathies* has been used to describe clinical disorders characterized by the presence of a microangiopathic hemolytic anemia (see Chapter 18 for more details on the pathophysiology of the hematologic aspects of these disorders), thrombocytopenia, and microvascular thrombotic lesions. **Disseminated intravascular coagulation (DIC)**, **thrombotic thrombocytopenic purpura (TTP)**, and **hemolytic uremic syndrome (HUS)** are TMA disorders. For a number of years, TTP and HUS were considered similar, perhaps overlapping, clinical conditions and were often included in a combined clinical classification of TTP/HUS. Both conditions are associated primarily with activation of platelets without activation of the coagulation cascade. Platelet aggregates form in the microvasculature and result in a thrombocytopenia. Typically, coagulation assays (PT, APTT, TT) are normal. It has recently been shown that the two disorders are distinct entities with different pathophysiologic causes, laboratory diagnostic approaches, and therapies (Table 35-10 ★).

Disseminated Intravascular Coagulation (DIC)

Also known as *consumptive coagulopathy*, DIC is a syndrome associated with systemic rather than localized activation of coagulation. It is a condition in which the normal balance of hemostasis is altered, resulting in the uncontrolled and inappropriate formation and lysis of fibrin within the blood vessels. It can be triggered by numerous events and can manifest as either a thrombotic or a hemorrhagic hemostatic state. See Chapter 34 for the full discussion of the pathophysiology and laboratory evaluation of patients with DIC.

★ **TABLE 35-10** Comparison of TTP and HUS

Feature	TTP	HUS
MAHA	Yes	Yes
Thrombocytopenia	Severe	Moderate to severe
Age	Peak incidence <40	Childhood
Gender	Female	Equal
Epidemic	No	Yes
Recurrence	Common	Rare
Link to *E. coli* O157:H7	Occasional	Yes
Renal failure	Uncommon	Common
Neurologic symptoms	Common	Uncommon
Organ involvement	Multiple	Limited to kidney
VWF multimer	Ultralarge forms present	Smaller multimers predominate
ADAMTS-13 activity	Deficient	Normal

TTP = thrombotic thrombocytopenic purpura; HUS = hemolytic uremic syndrome; MAHA = microangiopathic hemolytic anemia; VWF = von Willebrand factor

Thrombotic Thrombocytopenic Purpura (TTP)

Moschowitz first described TTP in his 1924 report of a 16-year-old female who presented with microangiopathic hemolytic anemia (MHA), petechiae, renal failure, and hemiparesis. At autopsy, she was found to have disseminated microvascular thrombi in the terminal arterioles and capillaries of the heart and kidney.[113] For years, TTP was defined by the "classic pentad" of (1) microangiopathic hemolytic anemia, (2) thrombocytopenia, (3) neurologic symptoms, (4) fever, and (5) renal dysfunction.[114] Because of the need for rapid treatment of this disorder using plasma exchange, less stringent diagnostic criteria are now typically applied. Currently, diagnosis can be made on the finding of MHA and thrombocytopenia in the absence of another clinically apparent cause.[115]

Pathophysiology. Systemic platelet agglutination produces microvascular thrombosis with variable tissue ischemia and infarction. The thrombi are composed predominantly of platelets with little or no fibrin. The most commonly affected organs are the brain, heart, pancreas, and adrenals; although renal involvement occurs, it rarely progresses to acute renal insufficiency or renal failure.

TTP is triggered by the presence of ultralarge VWF multimers (ULVWFs) in the plasma (Chapter 32). VWF is secreted from the Weibel-Palade bodies of activated endothelial cells as ultralarge multimers, larger than the typical large VWF forms that normally circulate. These ultralarge multimers are cleaved by a VWF cleaving protease; **a** **d**isintegrin-like **a**nd **m**etalloprotease with **t**hrombo**s**pondin type 1 motif (ADAMTS-13). ADAMTS-13 binds to the surface of endothelial cells and cleaves ULVWF multimers as they are synthesized and released[116,117] (Figure 35-5 ■). When ADAMTS-13 is significantly reduced or absent, the resulting ULVWF multimers can directly agglutinate platelets resulting in disseminated platelet thrombi.[115]

Clinical Presentation and Treatment. Familial or inherited TTP is known as the *Upshaw-Shulman syndrome*.[115] It is a rare autosomal recessive disorder characterized by repeated episodes of TMA during childhood (chronic, relapsing TTP). It is due to a mutation of the *ADAMTS-13* gene (9q34), resulting in a deficiency/dysfunction of the enzyme.[118] As a result, these patients have ULVWF in their plasma and a predisposition to forming platelet-rich microvascular thrombi. Treatment is plasma infusion to replace the missing enzyme as needed. Heterozygotes are clinically asymptomatic.

Acquired TTP is an acquired autoimmune deficiency of ADAMTS-13.[113] Also called *idiopathic TTP*, the disorder is more common in females than males (3:2 ratio). Other risk factors for idiopathic TTP are African ancestry and obesity. Of patients, 10–45% have a history of upper respiratory or flulike symptoms within weeks preceding diagnosis. Acquired TTP has also been associated with pregnancy, certain drugs, cancer/chemotherapy, and tissue transplantation. The autoantibodies against ADAMTS-13 block the activity of the VWF cleaving enzyme, producing TTP. Mortality is >90% without treatment. Recommended treatment is plasma exchange (to remove the autoantibody and replace with functional ADAMTS-13). Most patients (65%) have a single episode of TTP; ~35% have recurrent disease.

Laboratory Evaluation. Laboratory testing for ADAMTS-13 can be done to confirm the suspected diagnosis, but plasma exchange treatment should begin immediately without waiting for results.

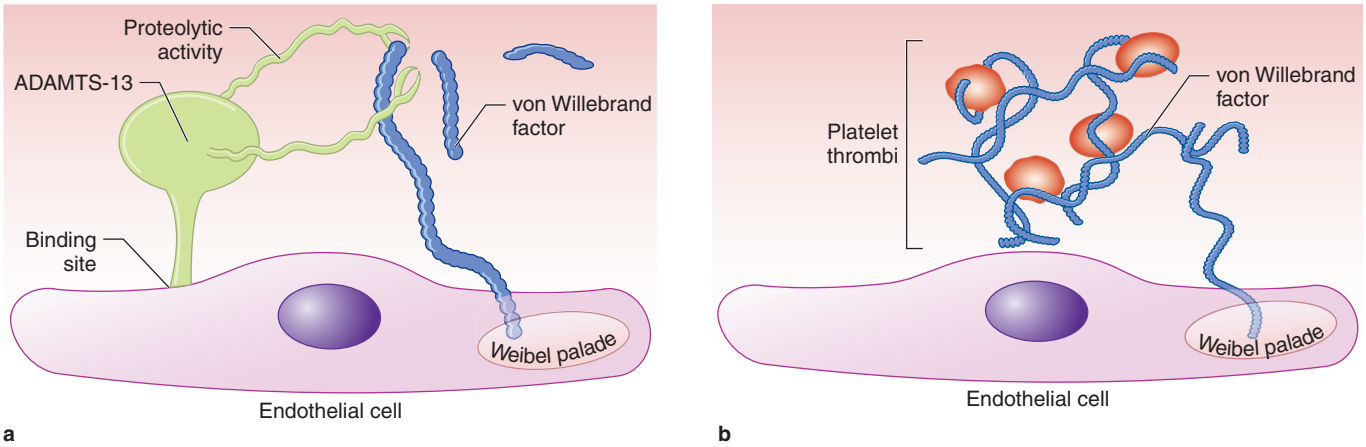


FIGURE 35-5 VWF processing by ADAMTS-13. **(a)** Normal ADAMTS-13 proteolytically cleaves ultralarge von Willebrand factor multimers (ULVWFs) into smaller subunits. **(b)** In the absence of ADAMTS-13 activity, ULVWF multimers persist, inducing platelet agglutination and the formation of platelet thrombi.

Schneider M. Thrombotic microangiopathy (TTP and HUS): advances in differentiation and diagnosis. *Clin Lab Sci.* 2007;20(4):216. Reprinted with permission from the American Society for Clinical Laboratory Science (ASCLS).

Most hospitals rely on reference laboratories for ADAMTS-13 assays. Antibody or inhibitor activity against the protease is measured by a Bethesda-type methodology similar to that used to measure antibodies to FVIII (Chapter 36).

Hemolytic Uremic Syndrome

Like TTP, HUS is a syndrome characterized by MHA and thrombocytopenia.

Pathophysiology. HUS occurs in two forms, typical and atypical.[115] Typical HUS is associated with bloody diarrhea, fever, and infection by verotoxin-producing *Escherichia coli* (VTEC). Also called *D+ HUS* (diarrhea positive), it accounts for 95% of all cases in children. *E. coli* O157:H7 accounts for most of the cases (80%), but other toxin-bearing *E. coli* serotypes and *Shigella dysenteriae* type 1 have also been linked with HUS.[119] The pathogenesis of VTEC-HUS is thought to be toxin-induced endothelial damage, primarily in the renal glomeruli, resulting in microvascular thrombi consisting of platelets and fibrin.

Familial (inherited) HUS is a chronic, relapsing clinical condition characterized as a D− or atypical form of HUS.[115] It is usually associated with an inherited abnormality of the complement regulatory system with persistently low levels of complement. Identified abnormalities include homozygous deficiency of complement factor H, membrane cofactor protein (MCP), or complement factor I. However, 50% of the cases of familiar HUS remain unexplained at the molecular level.

Clinical Findings. Thrombocytopenia and neurologic manifestations are less common and less severe in HUS than in TTP. However, renal involvement is more severe than in TTP. Of patients, 60% require renal dialysis, and 40–50% develop chronic renal insufficiency.

Treatment. Children with D+ HUS are usually managed by supportive care only. Adults may be considered for plasma exchange therapy because differentiation from TTP is sometimes uncertain. Plasma exchange may also be appropriate for individuals with inherited deficiencies of complement regulatory proteins.

Malignancy

The association between thrombosis and malignancy has been recognized for >100 years and for a variety of different cancers. Thrombotic disease occurs in 0.6–7.8% of cancer patients and is the second most common cause of death. Up to 50% of all cancer patients exhibit activation of the hemostatic system with elevated levels of markers of coagulation (e.g., thrombin/antithrombin complexes [TAT] prothrombin fragment 1.2, fibrinopeptide A, D-dimer).[120] Cancers with the highest thrombotic risk include tumors of the brain, pancreas, kidney, stomach, lung, and bladder and hematologic malignancies. The causes of thrombosis in cancer patients are complex. The three components that play a significant role in thrombogenesis (stasis, activation of blood coagulation, vascular injury) are present in most patients with malignant disease. Patients with cancer are often bedridden and immobile. Tumor cells release coagulation-activating factors including TF and cancer procoagulant protein (a cysteine protease that directly activates FX in the presence of FVIIa). Often malignancies are accompanied by a reduction of natural anticoagulants (AT, PC) or by defects of the fibrinolytic system. Surgery, chemotherapy drugs, and vascular access catheters can trigger vascular injury. Chemotherapy appears to augment the risk for thrombosis. Patients with malignancy also have a higher rate of postoperative thrombotic complications than the general population.[120] Plasma microparticles shed by platelets, endothelial cells, leukocytes, and the cancer cells themselves also contribute to the thrombotic risk in cancer patients. In some instances, thrombosis manifests before the diagnosis of an underlying malignancy and can predate the diagnosis by several years.[120]

Pregnancy and Oral Contraceptives (OCs)

Pregnancy is associated with an increased risk of VTE, and the puerperium (the 6-week period following delivery) is associated with a higher rate of thrombosis than pregnancy itself.[43] Risk factors for thrombosis in pregnancy include advanced maternal age, Cesarean delivery, prolonged immobilization, obesity, prior TE, APS, and one of the inherited thrombophilias. Thrombosis during pregnancy and the puerperium is the result of pregnancy-induced alterations in

hemostasis as well as venous stasis in the lower extremities. The levels of most coagulation proteins increase during pregnancy (particularly fibrinogen and FVIII), and AT, free and total PS, and fibrinolytic system activity (decreased tPA and increased PAI-1) significantly decline.[43] The net effect of these changes is to promote blood coagulation and may represent a mechanism to control bleeding at the time of placental separation.

Estrogen therapy and high-dose OCs have been associated with increased venous and arterial thrombosis related to the estrogen dose.[43] The mechanisms by which OCs induce a prothrombotic state are unclear. Their use is associated with changes in the levels of many coagulation proteins including increases of fibrinogen, FVII, and FVIII and decreases of AT and PS. Any individual with an inherited thrombophilia or increased thrombotic risk factors should be carefully evaluated before estrogen therapy is begun.

Postoperative State and Trauma

DVT and PE occur with increased frequency in postoperative patients although the thrombotic risk varies depending on the type of surgery performed. Risk factors associated with increased rates of thrombosis include age (advanced), previous VTE, coexistence of malignancy, inherited thrombophilia, and extended surgical and immobilization times.[11] DVT and PE are also commonly encountered after major trauma. Proposed mechanisms for the activation of the coagulation system include exposure of tissue factor from injured tissue, elevated levels of fibrinogen and VWF, decreased levels of AT and PC (associated with an acute inflammatory response), and increased numbers of circulating procoagulant microparticles.

Hematologic Disorders

Several hematologic disorders are associated with thrombosis. Patients with myeloproliferative disorders often experience a predisposition to thrombotic and hemorrhagic complications. Idiopathic myelofibrosis, essential thrombocythemia, and polycythemia vera are associated with venous and arterial thrombotic events. The mechanisms involved are complex and include the association of vascular occlusive episodes with a high hematocrit and hyperviscosity as well as elevated platelet counts and platelet hyper-reactivity (Chapter 24). Sickle cell anemia can lead to thrombosis because of hyperviscosity secondary to sickled erythrocytes. Acute promyelocytic leukemia (APL) is often complicated by a profound coagulopathy because of enhanced procoagulant activity associated with the release of TF from the leukemic cells (Chapter 26). Either diffuse or localized thrombosis can result. In paroxysmal nocturnal hemoglobinuria (PNH), the chronic intravascular hemolysis is often associated with thrombotic crises (Chapter 17). Membrane vesicles produced by complement-induced hemolysis of the erythrocytes possess prothrombinase-promoting activity and can induce thrombotic events at various vascular sites.

LABORATORY TESTING IN PATIENTS WITH SUSPECTED THROMBOSIS

When patients are suspected of having an acute thrombotic process or a thrombotic tendency, it is helpful to ascertain whether they are likely to have an inherited (primary) or an acquired (secondary) thrombophilia. Patients with hereditary defects are at lifelong risk of developing thrombosis, and clinical circumstances (e.g., pregnancy, estrogen use, surgery) can trigger thrombotic episodes in ~50% of such individuals.[43] Taking a complete history is essential, including age of onset, location of prior thromboses, circumstances that could have precipitated the event, and particularly a family history of other affected individuals (suggesting a hereditary defect).

Samples for laboratory analysis should not be drawn during a thrombotic episode because the episode itself can affect many laboratory assays. Acute thrombosis is often associated with acquired deficiencies of AT, PC, or PS because these proteins are consumed during thrombosis. Any anticoagulant therapy should be noted because it can affect coagulation tests. Heparin therapy can be associated with up to a 30% decline in AT levels, and warfarin produces a marked drop in the functional activity of PC and PS and a decline to a lesser extent in immunologic levels. Warfarin has also been shown to (rarely) elevate AT levels significantly, sometimes into the normal range in patients with a hereditary deficiency.[43]

Clinical guidelines for thrombotic risk assessment should be based on outcomes assessment. Many institutions have testing protocols for effective diagnosis in patients with thrombophilia. The protocol described here varies depending on the patient population, laboratory resources, and other factors (Figure 35-6 ■).

Purpose	Tests
Screening to rule out anticoagulant therapy and acquired factor deficiencies; detect antiphospholipid antibodies	PT, APTT
Common tests performed in a thrombotic risk battery	Assays for: • Protein C • Protein S • AT • APCR • Homocysteine • Fibrinogen disorders
Other tests as determined by institutional protocol	• Molecular assay for prothrombin G20210A • Assay for factor VIII increase • Tests for other Serpin deficiencies • Evaluation of fibrinolytic proteins TPA, PAI, α_2-plasmin inhibitor • Factor XII assay
Tests for concurrent thrombosis (for diagnostic purposes and to monitor thrombolytic therapy)	• D-dimer • Immunoassays for TAT complex, fibrinopeptide A and prothrombin fragment 1.2

■ **FIGURE 35-6** Protocol for thrombotic risk testing. Testing begins with coagulation screening tests and proceeds according to the protocol established by the institution.

Serpin = serine proteinase inhibitors (antithrombin, heparin cofactor II); TPA = tissue plasminogen activator; PAI = plasminogen activator inhibitor; AT = antithrombin; PT = prothrombin time; APTT = activated partial thromboplastin time; APCR = activated protein C resistance; TAT = thrombin-antithrombin complex

Thrombosis risk testing should begin with screening tests (PT, APTT) to rule out anticoagulant therapy and acquired factor deficiencies and to detect antiphospholipid antibodies that interfere with interpretation of clot-based PS and APCR tests. Additional testing typically includes functional and antigenic (or molecular) measurements of the most common inherited defects: APCR/FVL, prothrombin 20210, MTHFR mutations, PC, PS, and AT. Additional testing for other deficiencies or defects can be performed even if the patient tests positive for one of these initial tests because patients with thrombophilia can have multiple defects.

The best screening tests for deficiencies of AT, PC, and PS are functional assays that detect both quantitative and qualitative defects. Immunologic (antigenic) assays detect only quantitative deficiencies of these proteins. To obtain a definitive diagnosis (differentiate type I and type II deficiencies), thrombotic patients need to have both a functional and an antigenic test performed on the hemostatic component in question.

Many thrombotic disorders are difficult to diagnose because individuals can present with borderline laboratory values of a hemostatic component that are not clearly normal or abnormal; multiple testing can be needed. In addition to assays for particular hemostatic proteins associated with hereditary or acquired thrombophilias, assays are available to measure the extent of coagulation enzyme activation in the blood in vivo (e.g., fibrinopeptide A, prothrombin fragment 1.2, thrombin-AT complex, plasmin-antiplasmin complex). Although these assays do not give information regarding the specific *type* of hypercoagulable state that is present, they can provide information supporting the presence of increased activation of the coagulation system. Determining an episode's etiology is important because it suggests specific and appropriate therapy. All risk factors or possible deficiencies need to be ascertained because they can interact and, in some cases, significantly increase the risk of thrombosis. See Table 35-11 ★ for some tests that can be useful in diagnosing thrombotic disorders.

☑ CHECKPOINT 35-6

Why are functional assays recommended when screening a patient suspected of having a familial thrombophilic defect?

CASE STUDY *(continued from page 733)*

Initial screening tests left the physician with no clear explanation for the thrombotic events. Several years later, when new tests became available, Andrea, her mother, and two sisters were tested for the factor V Leiden mutation and prothrombin mutation 20210. Results follow:

	Mother	Andrea	Sister 1	Sister 2
FVL mutation	N	Heterozygous	N	N
20210 Mutation	Heterozygous	Heterozygous	Heterozygous	Heterozygous

3. Why is Andrea at greater risk for a thrombotic event than her mother or her two sisters?

★ **TABLE 35-11** Laboratory Tests Used to Diagnose Thrombophilia

	Functional	Antigenic	Molecular
Antithrombin	X	X	
Heparin cofactor II	X	X	
Protein C	X	X	
Protein S		Total PS	
	Free PS	Free PS	
APCR	X		X
Prothrombin 20210			X
Tissue factor pathway inhibitor	X	X	X
Hyperhomocysteinemia			
CBS mutations			X
MTHFR mutations			X
Fibrinogen	X	X	
Factor XII	X		
Plasminogen	X	X	
Tissue plasminogen activator	X	X	
Plasminogen activator inhibitor	X	X	
Antiphospholipid antibodies	Screening with APTT; specific confirmatory tests including platelet neutralization and dilute Russell's viper venom		

APCR = activated protein C resistance; CBS = cystathionine β synthase; MTHFR = methlene tetrahydrofolate reductase; APTT = activated partial thromboplastin time

ANTICOAGULANT THERAPY

The main objective of anticoagulant therapy is to treat or prevent thrombosis. Arterial thrombosis is often caused by the interaction of platelets and vessel wall atherosclerotic plaques and can be treated with antiplatelet drugs. **Thrombolytic therapy** can be useful in treating an acute arterial thrombosis because rapid clot lysis and restoration of blood flow minimize permanent tissue damage. The role of anticoagulant therapy in the treatment of arterial thrombosis is controversial.

Venous thrombosis is often associated with abnormalities in the plasma coagulation system. An acute DVT has historically been treated with heparin (or newer heparin-derived components) for several days followed by oral anticoagulant therapy for 3–6 months.

Heparin

Heparin is a heterogeneous group of molecules of sulfated glycosaminoglycans that bind to AT, resulting in rapid inhibition of serine proteases of the coagulation pathway. When administered parenterally, intravenously, or subcutaneously (heparin is not absorbed by the gastrointestinal tract), it produces a potent anticoagulant effect. Heparin does not have a direct effect on blood coagulation but facilitates AT's ability to neutralize serine proteases.

Mechanism of Action

Heparin for therapeutic use is usually extracted from porcine intestinal mucosa or bovine lung. The standard UFHs are heterogeneous mixtures of molecules with a mean molecular weight of ~15,000 Da (range 5000–30,000 Da). LMWH preparations (mean molecular weight of 4500–5,000 Da) are prepared from standard heparin by chemical or enzymatic depolymerization of UFH. Whereas UFH catalyzes AT inhibition of thrombin, FXa, and other serine proteases of the coagulation system, LMWH's major anticoagulant effect is to catalyze the interaction between AT and FXa. Recently, synthetic heparin pentasaccharides that mimic the active site of heparin have been produced. Fondaparinux, the first of this new group of drugs, also exerts its anticoagulant action by inhibiting FXa. By reducing the generation of or inactivating thrombin, these drugs prevent fibrin formation, inhibit thrombin-catalyzed activation of FV, FVIII, FXIIIa, and PC and inhibit thrombin activation of platelets.

The actions of two coagulation inhibitors are accelerated by heparin: AT and HCII. At therapeutic doses of heparin (0.2–0.4 U/mL), FIXa, FXa, and thrombin are inhibited almost exclusively by AT (which has a greater affinity for heparin than does HCII). In the presence of higher concentrations of heparin (or in the presence of dermatan sulfate), thrombin is inhibited also by HCII.

Dosage Considerations

The UFH dose required to produce a therapeutic effect varies from patient to patient because of differences in the plasma concentrations of various heparin-binding proteins (platelet factor 4, histidine-rich glycoprotein, fibronectin, VWF). These proteins bind to and neutralize large molecular weight heparins in the circulation. In some patients, larger doses of UFH than normal are required to achieve a therapeutic effect. The majority of these heparin-resistant patients have high levels of FVIII and heparin-binding proteins (which are acute-phase reactants) in their plasma. Patients with inherited AT deficiency (with AT levels of 40–60% of normal) respond normally to heparin therapy. However, individuals with acquired or inherited AT levels of $<$25% of normal can be resistant to the anticoagulant effects of heparin.

LMWH is being used with increasing frequency because of its convenience and more reliable pharmacokinetics.[121] The pharmacokinetic profile of UFH varies widely among individuals as a result of heparin binding to plasma proteins. LMWH and fondaparinux do not bind "heparin-binding proteins" in the plasma and thus have a more predictable dose-response profile; as a result, neither requires routine laboratory monitoring. Both have a longer half-life and a more predictable anticoagulant effect and are less likely to cause heparin-induced thrombocytopenia and osteoporosis.

Laboratory Monitoring

Several different laboratory tests can be used to determine proper UFH dosage. Individuals' response to UFH varies, so no standard dose provides protection from clotting while preventing adverse side effects. Therapy with UFH is usually monitored with a global test of coagulation, such as the activated clotting time (ACT) or the APTT. The PT is not sensitive to heparin and thus is not useful. Usually the heparin dose is adjusted so the APTT is 1.5–2.5 times the patient's pretreatment baseline value.[121] Because the sensitivities of APTT reagents and instruments vary widely, each clinical laboratory should determine its own heparin therapeutic range.

LMWH and fondaparinux typically do not require routine monitoring. However, in some circumstances (obesity, renal insufficiency, pediatric patients), monitoring may be indicated. LMWH and fondaparinux do not produce a predictable prolongation of the APTT at therapeutic doses, so if laboratory monitoring is required, an anti-Xa-based assay using a LMWH or fondaparinux standard should be performed.[121]

Complications

Heparin is an effective agent for treating and preventing VTE, but it can have adverse side effects. Excessive bleeding is the most common toxic effect of (high-dose) heparin therapy and has been reported in up to 30% of patients. HIT occurs in ~3% of patients receiving standard UFH but less frequently in patients receiving LMWH and very rarely in patients receiving fondaparinux (see the earlier section "Heparin-Induced Thrombocytopenia (HIT)"). Osteoporosis, or bone loss, is sometimes seen with long-term (1 month or more) standard heparin therapy. In contrast to warfarin, heparin does not cross the placenta and has not been associated with fetal malformations. Therefore, it is the anticoagulant of choice for prophylaxis and treatment of VTE during pregnancy. In cases of heparin overdose resulting in excessive anticoagulation or bleeding, protamine sulfate can be given to neutralize and reverse the anticoagulant effect of UFH. It has limited effect on neutralizing LMWH, and no effect on fondaparinux.

In the event that a patient develops HIT or is resistant to heparin, alternate anticoagulants can be used. These include direct anti-Xa inhibitors and direct thrombin inhibitors not requiring heparin (see section "New Anticoagulants").[121]

Oral Anticoagulants

Oral anticoagulants have been used to treat acute DVT and PE since the 1940s, and remain currently the most commonly used anticoagulants worldwide.[122] Coumadin drugs (e.g., sodium warfarin or dicoumarol) are vitamin K antagonists (VKAs) that inhibit coagulation by interfering with vitamin K's action in the liver. Coumadin inhibits hepatic carboxylation of the vitamin K-dependent proteins, resulting in the release of nonfunctional (incompletely γ-carboxylated) molecules to the plasma. These proteins have reduced functional activity relative to their antigenic levels.

Mechanism of Action

Coumadin blocks the vitamin K epoxide reductase enzyme that converts vitamin K epoxide back to the reduced form of vitamin K, which is the form required as a cofactor for the carboxylation reaction (Figure 32-3). The anticoagulant effect of Coumadin depends on the reduced synthesis of biologically active vitamin K-dependent proteins and the normal clearance from the circulation of fully active proteins synthesized before the introduction of the drug. Coumadin inhibits γ-carboxylation of newly synthesized proteins but does not affect the plasma half-life of already circulating proteins. The anticoagulant effect of Coumadin thus lags behind the point at which optimal plasma Coumadin concentration is reached. The disappearance of biologically active, fully γ-carboxylated factors is determined by the

half-life of each. FVII activity disappears most rapidly ($T_{1/2}$ ~6 hours), FX and FIX follow ($T_{1/2}$ ~24 hours), and prothrombin disappears at the slowest rate because of its longer half-life ($T_{1/2}$ ~72 hours).[122] Thus, Coumadin does not produce instantaneous anticoagulation but must be administered for 4–5 days before therapeutic anticoagulation is achieved. Coumadin is administered orally, is nearly completely absorbed, and circulates in the blood bound to albumin (97%). Only free Coumadin is biologically active. Its plasma half-life is ~36 hours.

Dosage Considerations

Patients who have had a thrombotic incident are normally treated with heparin during the initial phase of anticoagulation because it produces an immediate anticoagulant effect. Because Coumadin's full anticoagulant action is achieved only after 4–5 days, Coumadin and heparin are given simultaneously during this time. Subsequently, when oral anticoagulation has been achieved, heparin can be discontinued. Coumadin is usually the therapy of choice for chronic anticoagulation to prevent the recurrence of thromboembolic disease. After the treatment of an acute thrombosis (DVT or PE), patients are routinely maintained on Coumadin for 3–6 months.

Genetic factors can influence Coumadin's anticoagulant effect.[122] Variant alleles for either the *CYP2C9* (the gene coding for cytochrome P-450) or *VKORC1* (the gene encoding the vitamin K epoxide reductase) result in altered pharmacokinetics of Coumadin, and variable doses of the drug may need to be given to maintain a therapeutic level. Molecular testing can identify these mutations affecting Coumadin's response.

Laboratory Monitoring

The PT is routinely used to monitor VKA anticoagulation. The therapeutic Coumadin dosage varies among individuals, and monitoring the anticoagulant effect can be difficult. A major problem has been a variation in the potency of thromboplastins used by different manufacturers. The introduction of a reference standard for thromboplastins by the World Health Organization International Sensitivity Index and the use of the international normalized ratio (INR) have reduced the interlaboratory and interhospital variability in monitoring oral anticoagulants (Chapter 36).

Complications

Bleeding complications are observed in 10–20% of patients treated with Coumadin; about 50% of these complications occur when the therapeutic range of the PT has been exceeded, but 50% occur despite a PT within the therapeutic range. Although bleeding is usually mild, severe and life-threatening bleeding can occur. Thus, patients receiving oral anticoagulation must be carefully monitored. During the initiation of therapy, PT measurements can be required 2 or 3 times a week; once stabilized, patients generally require a PT every 3 or 4 weeks.

Thrombotic events can occasionally occur with Coumadin administration because of a transient disruption of the procoagulant/anticoagulant balance leading to microthrombosis. The pathogenesis is depletion of one or both vitamin-dependent anticoagulant proteins (PC, PS) more quickly than depletion of the procoagulant vitamin K proteins, resulting in a temporary hypercoagulable state. The most common clinical manifestation is Coumadin-induced skin necrosis.

The antidote for Coumadin overdose is administration of vitamin K. Coumadin crosses the placenta and has been associated with fetal abnormalities; therefore, its use is contraindicated during pregnancy.

New Anticoagulants

A number of new drugs that inhibit the initiation or propagation of coagulation have been developed and are in various stages of clinical trials and/or FDA approval.[123] These drugs are classified as direct or indirect inhibitors. Direct inhibitors bind directly to their target enzyme and block their interaction with substrate. Indirect inhibitors function by binding plasma cofactors, such as antithrombin, accelerating their interaction with clotting enzymes. Of particular interest are the new oral direct FXa inhibitors (e.g., rivaroxaban, apixaban, edoxaban) and direct thrombin inhibitors (e.g., dabigatran) anticoagulants.

Thrombolytic Therapy

Thrombolytic therapy is a clinical approach to TE disease with the goal of re-establishing vascular perfusion. All currently used thrombolytic agents are plasminogen activators (PA) used to lyse thrombi in vivo.[124] Clinical trials assessing efficacy have been conducted for DVT, PE, peripheral arterial occlusion, acute myocardial infarction (MI), and acute ischemic stroke. The potential clinical benefit of thrombolytic treatment for acute thrombosis is in part determined by the time frame in which treatment should be initiated. For MI or cerebral thromboses, treatment should be started within 4 and 3 hours, respectively, whereas treatment should be initiated for PE within 48 hours. Thrombolytic therapy for DVT maintains efficacy if treatment is initiated within 7 days.

Mechanism of Action

All of the PAs are capable of inducing plasmin lysis of fibrin within a thrombus and are accompanied by a variable degree of plasma fibrinogenolysis (the lytic state). Under normal physiologic conditions, plasminogen and PAs interact when bound to fibrin in the thrombus. Fibrin facilitates the conversion of plasminogen to plasmin because PAI-1 and α_2-antiplasmin (AP) do not efficiently inhibit fibrin-bound PA and plasmin, respectively. Under physiologic conditions, systemic lysis of fibrinogen does not occur because PAI-1 and AP efficiently inhibit tPA and plasmin in the circulation. With administration of therapeutic dosages of PA, virtually all of the plasma plasminogen is converted to plasmin, overwhelming the neutralizing capacity of AP and leading to some degree of systemic proteolysis.

Dosage Considerations

The FDA has approved six plasminogen activators: streptokinase/SK, urokinase/UK (uPA), alteplase/tPA, anistreplase, reteplase, and tenecteplase.[124] UK currently is not available in the United States, and anistreplase is rarely used. The major distinctions among the agents relate to their antigenicity, half-life, potential for inducing a lytic state, and hemorrhagic potential. Those PAs derived from a human protein (UK, tPA, alteplase, tenecteplase, and reteplase) are essentially nonantigenic whereas those from a bacterial species can induce antibody formation. The agents' half-lives vary from 5 minutes for tPA to 20 minutes for SK to 70 minutes for anistreplase. Several new PAs are in various stages of development or clinical trials. The continued

search for new PA variants seeks to develop new agents with increased activity, a longer half-life, or a reduced lytic state.

Laboratory Monitoring

The *lytic state* occurs because of the systemic conversion of plasminogen to plasmin by circulating PA and the effects of plasmin on components of the circulating blood. Although a number of plasma proteins are degraded, the identification of the lytic state is usually a demonstration of a decrease in plasma fibrinogen.[124] This increased degradation of fibrinogen is detected by a prolonged thrombin time test and increased fibrinogen degradation products (FDPs). Plasmin also degrades other plasma proteins including FV and FVIII. Other potential laboratory tests to monitor the lytic state include a shortened euglobulin lysis time, a quantitative decrease in circulating plasminogen and α_2-antiplasmin, and the generation of plasmin-antiplasmin complexes (PAP). Free circulating plasmin can be demonstrated using chromogenic assays. Free plasmin also affects platelet function by decreasing aggregation induced by various platelet agonists and by cleaving the platelet membrane receptor for VWF. The lytic state induces a hypocoagulable state resulting from a combination of decreased clottable fibrinogen and other procoagulant proteins, the generation of FDPs (which inhibit coagulability), and platelet hyporeactivity.[124]

PA dosage regimens are standardized, thus, monitoring thrombolytic therapy is necessary only to document that a lytic state has indeed been achieved.[124] The TT is sometimes used to monitor fibrinolytic therapy. A baseline TT should be determined and another performed at 3–4 hours after starting therapy. If a lytic state is induced, the TT should become prolonged because of the increased fibrin(ogen) degradation products and decreased fibrinogen level. FDP or D-dimer assays can be used to determine whether the thrombus is lysing.

Complications

The degradation of fibrin produces the beneficial effect of reducing the size of the thrombus (thrombolysis). However, a potential complicating effect can be the lysis of hemostatic plugs and resultant bleeding. Blood hypocoagulability can compound the bleeding tendency because of fibrinogenolysis and platelet dysfunction caused by the increased lytic state. The patient's response to PA therapy is inadequate if the thrombosed vessel does not manifest full reperfusion or if reocclusion quickly follows initial success. Thus, adjunctive treatment with anticoagulants and/or antiplatelet agents is often added to thrombolytic therapy to minimize early reocclusive events.[124]

Antiplatelet Therapy

Various antiplatelet agents are available to reduce or block platelet responsiveness and activation. As with anticoagulant therapy, antiplatelet therapy includes the use of both oral and intravenous agents. Laboratory monitoring generally is not necessary with antiplatelet agents except for the need to monitor platelet counts with some of the drugs that cause thrombocytopenia in a small number of patients.

Aspirin

Aspirin is probably the most common drug used as an antithrombotic/antiplatelet agent in managing arterial thrombosis. Although it has been used for more than a century as an analgesic and antipyretic, it has been used as an antithrombotic only since the 1950s.[125] Aspirin blocks prostaglandin synthesis (TXA_2) in platelets by permanently and irreversibly inhibiting cyclooxygenase (COX-1 enzyme) in them. Aspirin accomplishes this by acetylating platelet COX-1, blocking the enzyme's ability to interact with its substrate arachidonic acid. As little as a single "baby aspirin" (~30 mg) per day is used as prophylaxis for stroke (far below the dosage used for pain and fever). The unique sensitivity of platelets to aspirin's therapeutic effect results from the fact that platelets are cytoplasmic fragments from megakaryocytes and lack a nucleus and ribosomes. As a result, the platelet cannot replace its aspirin-acetylated, permanently inhibited COX-1 by synthesis of new proteins and continues to circulate for the rest of its life span in an inactive/inactivatable state (~9–10 days). In spite of the fact that aspirin also inhibits COX-1 in endothelial cells, blocking PGI_2 (platelet inhibitor) production, it has a net antithrombotic effect because these nucleated cells simply synthesize new COX-1 to replace the aspirin-inhibited enzyme. Patients taking aspirin do not have a severe bleeding diathesis, and aspirin exerts only a modest effect on platelet function and bleeding times. The prostaglandin-TXA_2 pathway augments platelet aggregation to weak agonists but is not needed for platelet responsiveness to strong agonists such as ADP or thrombin.[125]

Other NSAIDS

The nonsteroidal anti-inflammatory drugs (NSAIDs), excluding aspirin, are competitive inhibitors of COX-1 (and COX-2) rather than irreversible inhibitors. Because their actions block enzyme function transiently, they are not generally used as antithrombotic agents alone but sometimes in conjunction with aspirin. NSAIDs include ibuprofen, flurbiprofen, and sulfinpyrazone. However, because they do have an antiplatelet effect although transient, surgeons recommend that patients not take these drugs for 7–10 days before surgery (as with aspirin). Acetaminophen does not affect platelet function.

ADP Receptor Antagonists

Adenosine diphosphate (ADP) receptor antagonists—ticlopidine, clopidogrel and prasugrel—are drugs that block a different platelet activation pathway than do aspirin and other NSAIDs. They modify the ADP receptor, blocking platelet $P2Y_{12}$ receptor activity.[107] These drugs have been linked to causing TTP in a small number of patients. Ticlopidine is also associated with neutropenia and marrow suppression in a small number of patients (1–2%), which clopidogrel does not. Clopidrogrel has a lower incidence of TTP and thus is generally the ADP receptor-antagonist drug of choice for most patients.

Dipyridamole

Dipyridamole inhibits phosphodiesterase, resulting in increased cAMP levels and inhibition of platelet activation. It has been used in patients for decades but has limited antithrombotic effectiveness when used alone. It is often combined with aspirin to improve effectiveness.

Intravenous Antiplatelet Agents

Intravenous antiplatelet agents include inhibitors of GPIIb/IIIa. The GPIIb/IIIa platelet membrane receptor interacts with fibrinogen and VWF during platelet activation and aggregation. In contrast to the previously discussed drugs, which block only one of multiple platelet activation pathways, the blockade of GPIIb/IIIa

almost completely eliminates platelet function if given in sufficient dosages. Abciximab (ReoPro) has been used for a number of years although its use requires the platelet count to be monitored because it causes thrombocytopenia in 1–2% of treated patients. Eptifibatide (Integrilin) has also been used and is generally not associated with thrombocytopenia.

☑ CHECKPOINT 35-7

Why should heparin therapy overlap initiation of oral anticoagulant therapy when treating a patient with an acute thrombosis?

Summary

The human hemostatic system normally maintains a balance between procoagulant or thrombogenic factors and anticoagulant or fibrinolytic activity. If this delicate balance is disturbed, the result can be excessive bleeding or unwanted clotting. Arterial and/or venous thrombosis occurs when activation of blood coagulation exceeds the ability of the natural protective mechanisms (anticoagulants/inhibitors and the fibrinolytic system) to prevent or minimize fibrin formation. Clinical risk factors that predispose an individual to either arterial or venous thrombosis have been identified. Thrombophilia or hypercoagulability refers to any disorder (inherited or acquired) with an increased tendency to venous thromboembolism. Familial thrombophilia generally results from three broad categories of hemostatic abnormalities: (1) accelerated fibrin formation (increased procoagulant activity or diminished natural inhibitor activity), (2) defective fibrinolysis (reduced profibrinolytic factors or increased fibrinolytic inhibitors), or (3) abnormal fibrin (dysfibrinogenemias). An inherited thrombophilic defect places an individual at risk for but does not inevitably lead to thrombosis. Thrombosis occurs in susceptible patients who have one or more genetic mutations when they are exposed to exogenous prothrombotic stimuli. The five most common and most clearly delineated hereditary abnormalities associated with thrombophilia are factor V Leiden, prothrombin 20210, and decreased or defective antithrombin, PC, and PS.

Patients with a documented thrombotic event need to be carefully evaluated including a complete personal and family history. Any additional risk factors for thrombosis present at the time of the incident (e.g., surgery, pregnancy, venous stasis) should be assessed. If a positive family history exists, the thrombosis was spontaneous, or multiple thrombotic incidences occurred, a full diagnostic workup should be undertaken to ascertain, if possible, the etiology of the thrombotic event. The evaluation of many of the components that regulate hemostasis (procoagulant proteins, inhibitory proteins, and fibrinolytic regulators) requires both functional (activity) and quantitative (immunologic) assays because both qualitative and quantitative defects of these proteins occur. Definitive diagnosis of some inherited thrombophilic disorders requires molecular analysis of the implicated gene. The presence of multiple inherited or acquired thrombophilic factors significantly increases the risk for thrombosis. Patients experiencing an acute thrombotic event can be treated with a thrombolytic agent (plasminogen activators), heparin or low-molecular-weight heparin, or oral anticoagulants. Long-term anticoagulation usually utilizes oral anticoagulants. Antiplatelet therapy can be used to manage arterial thrombosis. These agents block platelet responsiveness and activation. The most common antiplatelet prophylaxis is aspirin. Individuals with inherited thrombophilia usually do not require prophylactic therapy unless an additional prothrombotic clinical risk factor exists (prolonged immobility, surgery, pregnancy, etc.).

Review Questions

Level I

1. A platelet fibrin mass that forms within a vessel is known as a(n): (Objective 1)
   A. thrombus
   B. embolism
   C. platelet plug
   D. clot

1. Rupture of plaque in an artery can result in: (Objective 4)
   A. the formation of a white thrombi
   B. the formation of a red thrombi
   C. hyperhomocysteinemia
   D. decreased fibrinolysis

3. A patient is diagnosed with DVT. Four days later, a thrombus is found in his lung. This is an example of: (Objectives 3, 5)
   A. a white thrombi
   B. local thrombosis
   C. a thromboembolus
   D. DIC

4. Which type of laboratory test results for AT will a patient with a type I deficiency of AT have? (Objective 8)
   A. decreased antigenic and normal functional activity
   B. normal antigenic and normal functional activity
   C. normal antigenic and decreased functional activity
   D. decreased antigenic and decreased functional activity

5. An inherited abnormality in the factor V molecule that renders it resistant to inactivation by APC is known as: (Objective 9)
   A. AT deficiency
   B. APCR
   C. DIC
   D. prothrombin G20210A
6. A deficiency or defect in PC can lead to thrombosis because of an: (Objective 7)
   A. inability to inactivate PS
   B. increased platelet activation
   C. inability to neutralize thrombin
   D. inability to inactivate factors Va and VIIIa
7. Patients who receive UFH as treatment for a thrombus should be monitored periodically for the complication of HIT using what laboratory test? (Objective 10)
   A. APTT
   B. PT/INR
   C. TT
   D. platelet count
8. Therapy with LMWH is best monitored using which laboratory test? (Objectives 10, 11)
   A. PT/INR
   B. APTT
   C. factor Xa assay
   D. platelet count
9. The best test to monitor Coumadin therapy is the: (Objective 12)
   A. PT/INR
   B. APTT
   C. factor Xa assay
   D. platelet count
10. What physiologic protein's anticoagulant effect does heparin accelerate? (Objective 6)
    A. AT
    B. protein C
    C. protein S
    D. lupus anticoagulant

Level II

1. Which of the following clinical manifestations is more likely to be found in a person with an inherited thrombophilia than in a person with an acquired thrombophilia? (Objectives 2, 10)
   A. venous embolism at a young age
   B. absence of a family history of thrombosis
   C. presence of a pulmonary embolism
   D. myocardial infarction in a 50-year-old
2. A patient with thrombophilia has a decreased functional and antigenic activity of PC. What is the diagnosis? (Objective 4)
   A. APCR
   B. PS deficiency
   C. type I PC deficiency
   D. type II PC deficiency
3. Why is following up an abnormal clotting assay for APCR with a molecular test recommended? (Objective 6)
   A. The clotting test is not sensitive or specific for APCR.
   B. Of individuals with APCR, 10% do not have the FVL mutation.
   C. The clotting test cannot be used for 6 months after a thrombotic episode.
   D. The molecular test is inexpensive and more accurate.
4. Why does a patient with a thrombotic incident receive both heparin and Coumadin for 4–5 days after the incident? (Objective 11)
   A. Heparin is not effective as an anticoagulant without Coumadin present.
   B. Coumadin requires heparin for its full anticoagulant effect.
   C. Doing so gives the patient an initial bolus dose of anticoagulant.
   D. Coumadin takes this long to produce its full anticoagulant action.
5. The value of using the INR to report the PT is that: (Objective 12)
   A. it reduces interlaboratory variability in monitoring oral anticoagulants
   B. it can be used to monitor both heparin and Coumadin therapy
   C. patients on Coumadin therapy do not need to be tested as often
   D. patients' Coumadin dosage does not need to be adjusted as frequently

6. A patient with a myocardial infarction is admitted to the ER. The physician starts the patient on streptokinase and calls the laboratory and wants you to suggest a test to ensure that a lytic state is induced by the therapy. What test will you suggest? (Objective 13)

   A. a baseline TT and TT after 3–4 hours of therapy

   B. fibrinogen, plasminogen, and plasmin assays

   C. a PT, APTT, and platelet count

   D. none because no available tests give this information

7. A patient has hyperhomocysteinemia. Which laboratory test(s) can be helpful in establishing the etiology of the disease? (Objective 7)

   A. APCR clotting assay

   B. AT and PS antigenic assays

   C. molecular tests for MTHFR and CBS

   D. molecular test for prothrombin G20210A

8. Which of the following conditions is *not* associated with an increased tendency for thrombosis? (Objective 10)

   A. antiphospholipid antibody syndrome

   B. pregnancy

   C. malignancy

   D. factor VIII deficiency

9. A 30-year-old patient is diagnosed with his third episode of DVT. He is currently hospitalized and receiving heparin therapy. The physician orders a thrombotic risk battery of tests. The most appropriate action that the laboratory should take is to: (Objective 1)

   A. call the physician and explain that testing will not be accurate during anticoagulant therapy and during the thrombotic episode

   B. perform the battery of tests in the thrombotic risk profile but note that results are not reliable

   C. perform a PT and an APTT and, if prolonged, refuse to do the testing

   D. call the physician and explain that this patient is not a candidate for thrombotic risk testing

10. A patient on Coumadin for treatment of DVT returns to the doctor with skin necrosis. What protein deficiency should be tested for after Coumadin therapy is finished? (Objective 5)

    A. AT

    B. PC

    C. HCII

    D. TPAI

Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

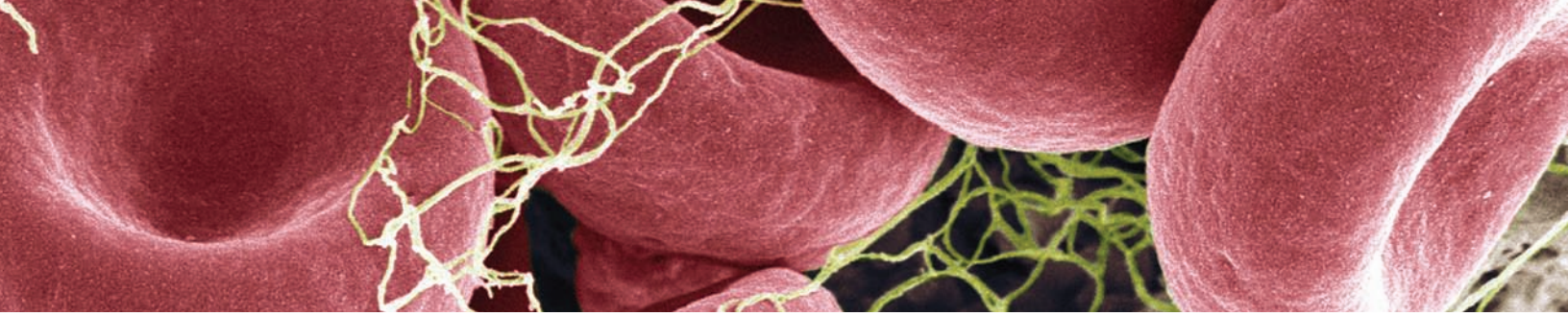
References

1. Schulman S, Marder VJ. Overview of arterial thrombotic disorders. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1043–46.
2. Libby P, Ridker PM, Croce K. Pathophysiology of atherothrombosis. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1047–63.
3. Silverstein RL. Atherothrombosis. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone/Elsevier; 2013:2057–66.
4. Ridker PM, Stampfer MJ, Rifai N. Novel risk factors for systemic atherosclerosis: a comparison of C-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. *JAMA*. 2001;285:2481–485.
5. Kaptoge S, Di Angelantonio E, Lowe G et al. Emerging risk factors collaboration: C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet*. 2010;375:132–40.
6. Ridker PM, Rifai N, Rose L et al. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med*. 2002;347(20):1557–565.
7. Ridker PM, Glynn RJ, Henneker CH. C-reactive protein adds to the predictive value of total and HDL cholesterol in determining risk of first myocardial infarction. *Circulation*. 1998;97:2007–11.
8. Flinterman LE, Van Der Meer FJ, Rosendaal FR et al. Current perspective of venous thrombosis in the upper extremity. *J Thromb Haemost*. 2008;6:1262–266.
9. Schulman S, Marder VJ, White GC. Overview of venous thromboembolism. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:957–61.
10. Lim W. Venous thromboembolism. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Churchill Livingstone/Elsevier; 2013:2039-47.
11. Heit JA. Epidemiology and risk factors for venous thromboembolism. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:973–76.

12. Heit J, Mohr D, Silverstein M et al. Predictors of recurrence after deep vein thrombosis and pulmonary embolism: a population-based cohort study. *Arch Intern Med*. 2000;160:761–68.
13. Heit J, O'Fallon W, Petterson T et al. Relative impact of risk factors for deep vein thrombosis and pulmonary embolism: a population-based study. *Arch Intern Med*. 2002;162:1245–248.
14. Noboa S, Mottier D, Oger E, EPI-GETBO Study Group. Estimation of a potentially preventable fraction of venous thromboembolism: a community-based prospective study. *J Thromb Haemost*. 2006;4:2720–722.
15. Pandolfi M, Robertson B, Isacson S et al. Fibrinolytic activity of human veins in arms and legs. *Thromb Diath Haemorrh*. 1968;20:247–56.
16. Jick H, Westerholm B, Vessey MP et al. Venous thromboembolic disorder and ABO blood type: a cooperative study. *Lancet*. 1969;293:539–42.
17. Wautrecht JC, Galle C, Motte S et al. The role of ABO blood groups in the incidence of deep vein thrombosis. *Thromb Haemost*. 1998;79:668–69.
18. Larsen TB, Johnsen SP, Gislum M et al. ABO blood groups and risk of venous thromboembolism during pregnancy. *J Thromb Haemost*. 2005;3:300–304.
19. Schleef M, Strobel E, Dick A et al. Relationship between ABO and secretor genotype with plasma levels of factor VIII and von Willebrand factor in thrombosis patients and control individuals. *Br J Haematol*. 2004;128:100–107.
20. Morelli VM, DeVisser MC, Vos HL et al. ABO blood group genotypes and the risk of venous thrombosis: effect of factor V Leiden. *Thromb Haemost*. 2005;3:183–85.
21. Franchin M, Capra F, Targher G et al. Relationship between ABO blood group and von Willebrand levels: from biology to clinical implications. *Thromb J*. 2007;5:14–19.
22. Bounameaux H, Perrier A. Clinical manifestations and diagnosis of venous thromboembolism. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:977–94.
23. Bounameaux H, DeMoerloose P, Perrier A et al. Plasma measurement of D-dimer as a diagnostic aid in suspected venous thromboembolism: an overview. *Thromb Haemost*. 1994;71:1–6.
24. Wells PS, Brill-Edwards P, Stevens P et al. A novel and rapid whole-blood assay for D-dimer in patients with clinically suspected deep vein thrombosis. *Circulation*. 1995;91:2184–187.
25. Burnier L, Fontana P, Kwak BR et al. Cell-derived microparticles in haemostasis and vascular medicine. *Thrombosis Haemost*. 2009;101:439–51.
26. Dignat-Geoerge F, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol*. 2011;31:27–33.
27. Lacroix R, Dubois C, Leroyer AS et al. Revisited role of microparticles in arterial and venous thrombosis. *J Thromb Haemost*. 2013;11(suppl 1):24–35.
28. Freyssinet JM, Dignat-George F. More on: measuring circulating cell-derived microparticles. *J Thromb Haemost*. 2005;3:613–14.
29. Davizon P, Lopez JA. Microparticles and thrombotic disease. *Curr Opin Hematol*. 2009;16:334–41.
30. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. *Circ Res*. 2010;107:1047–57.
31. Tushuizen ME, Diament M, Sturk A et al. Cell-derived microparticles in the pathogenesis of cardiovascular disease: friend or foe? *Arterioscler Thromb Vasc Biol*. 2011;31:4–9.
32. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. *Blood Rev*. 2007;21:157–71.
33. van der Pol E, Coumans F, Varga Z et al. Innovation in detection of microparticles and exosomes. *J Thromb Haemost*. 2013;11(suppl 1):36–45.
34. World Health Organization Fact Sheet. www.who.int/mediacentre/factsheets/fs310/en/; updated July 2013.
35. Brenner, B, Aharon A, Gris J-C. Gestational vascular complications. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1393–1401.
36. Schafer AI. Hypercoagulable states: molecular genetics to clinical practice. *Lancet*. 1994;344:1739–742.
37. Middeldorp S, Buller HR, Prins MH et al. Approach to the thrombophilic patient. In: Colman RW, Hirsh J, Marder VJ et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2000:1085–1100.
38. Marder VJ, Matei DE. Hereditary and acquired thrombophilic syndromes. In: Colman RW, Hirsh J, Marder VJ et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Pratice*, 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2000:1243–275.
39. Emmerich J, Aiach M, Morrange P-E. Thrombophilia genetics. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:962–72.
40. Morange P-E, Tregouet D-A. Current knowledge on the genetics of incident venous thrombosis. *J Thromb Haemost*. 2013;11(suppl 1):111–21.
41. Egeberg O. Inherited antithrombin deficiency causing thrombophilia. *Thromb Diath Haemorrh*. 1965;13:516.
42. Thaler E, Lechner K. Antithrombin III deficiency and thromboembolism. In: Prentice CRM, ed. *Clinics in Haematology*. Vol 10. London: Saunders; 1981:369–90.
43. Bauer KA. Hypercoagulable states. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Procedures*, 3rd ed. New York: Churchill Livingstone; 2000:2009–39.
44. Bucciarelli P, Passamonti SM, Biguzzi E et al. Low borderline plasma levels of antithrombin, protein C and protein S are risk factors for venous thromboembolism. *J Thromb Haemost*. 2012;10:1783–791.
45. Anton AI, Teruel R, Corral J et al. Functional consequences of the prothrombotic SERPINC1 rs2227589 polymorphism on antithrombin levels. *Haematologica*. 2009;94:589–92.
46. Bezmer ID, Bare LA, Doggen CJ et al. Gene variants associated with deep vein thrombosis. *JAMA*. 2008;299:1306–314.
47. Vinazzer H. Hereditary and acquired antithrombin deficiency. *Semin Thromb Hemost*. 1999;25:257–63.
48. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc Natl Acad Sci U S A*. 1981;78:2249–252.
49. Esmon CT. Protein C, protein S, and thrombomodulin. In: Colman RW, Hirsh J, Marder VJ et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2000:335–53.
50. Nizzi F, Kaplan H. Protein C and S deficiency. *Semin Thromb Hemost*. 1999;25:265–72.
51. Griffin J, Evatt B, Zimmerman T et al. Deficiency of protein C in congenital thrombotic disease. *J Clin Invest*. 1981;68:1370–373.
52. Dennis J, Johnson CY, Adediran AS et al. The endothelial protein C receptor (PROCR) Ser210Gly variant and risk of common thrombotic disorders: a HuGE review and meta-analysis of evidence from observational studies. *Blood*. 2012;119:2392–400.
53. Casella JF, Lewis JH, Bontempo FA et al. Successful treatment of homozygous protein C deficiency by hepatic transplantation. *Lancet*. 1988;1:435–38.
54. McGehee WG, Klotz TA, Epstein P et al. Coumarin necrosis associated with hereditary protein C deficiency. *Ann Int Med*. 1984;101:59–60.
55. Mosnier LO, Griffin JH. Protein C, protein S, thrombomodulin and the endothelial protein C receptor. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:300–13.
56. Comp PC, Nixon RR, Cooper MR et al. Familial protein S deficiency is associated with recurrent thrombosis. *J Clin Invest*. 1984;74:2082–88.
57. Schwarz HP, Muntean W, Watzke H et al. Low total protein S antigen but high protein S activity due to decreased C4B-binding protein in neonates. *Blood*. 1988;71:562–65.
58. Brunet D, Barthet MC, Morange PE et al. Protein S deficiency: different biological phenotypes according to the assays used. *Thromb Haemost*. 1998;79:446–47.
59. Dahlbäck B, Carlsson M, Svensson P. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C. *Proc Natl Acad Sci U S A*. 1993;90:1004–8.
60. Dahlbäck B. Activated protein C resistance and thrombosis: molecular mechanisms of hypercoagulable state due to FVR506Q mutation. *Semin Thromb Hemost*. 1999;25:273–89.
61. Castoldi E, Brugge JM, Nicolaes GA et al. Impaired APC-cofactor activity of factor V plays a major role in the APC resistance associated with the factor V Leiden (R506Q) and R2 (H1299R) mutations. *Blood*. 2004;103:4173–179.
62. Bernard F, Faioni E, Castoldi E et al. A factor V genetic component differing from factor VR506Q contributes to the activated protein C resistance phenotype. *Blood*. 1997;90:1552–557.

63. Williamson D, Brown K, Luddington R et al. Factor V Cambridge: a new mutation (Arg 306 → Thr) associated with resistance to activated protein C. *Blood*. 1998;91:1140–144.
64. Chan WP, Lee CK, Kwong YL et al. A novel mutation of Arg 306 of factor V gene in Hong Kong Chinese. *Blood*. 1998;91:1135–139.
65. Norstrom E, Thorelli E, Dahlback B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. *Blood*. 2002;100(2):524–30.
66. Castoldi E, Rosing J. APC resistance: biological basis and acquired influences. *J Thromb Haemost*. 2010;8(3):445–53.
67. Kaufman RJ, Fay PJ, Popolo L et al. Factor V and factor VIII. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:179–96.
68. Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet*. 1995;346:1133–134.
69. Poort S, Rosendaal F, Reitsma P et al. A common genetic variation in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood*. 1996;88:3698–703.
70. Martinelli I, Battaglioli T, Tosetto A et al. Prothrombin A19911G polymorphism and the risk of venous thromboembolism. *J Thromb Haemost*. 2006;4:2582–586.
71. Markis M, Preston FE, Beauchamp NJ et al. Co-inheritance of the 20210 allele of the prothrombin gene increases the risk of thrombosis in subjects with familial thrombophilia. *Thromb Haemost*. 1997;78:1426–429.
72. Villa P, Azner J, Vaya A et al. Hereditary homozygous heparin cofactor II deficiency and the risk of developing venous thrombosis. *Thromb Haemost*. 1999;82:1011–14.
73. Tollefsen DM. Heparin cofactor II deficiency. *Arch Pathol Lab Med*. 2002;126:1394–400.
74. Rapaport SI. The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coaglation. *Thromb Haemost*. 1991;66:6–15.
75. Sandset PM. Tissue factor pathway inhibitor (TFPI): an update. *Haemostasis*. 1996;26:154–65.
76. Dahm A, Van Hylckama Vlieg A, Bendz B, et al. Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. *Blood*. 2003;101:4387–392.
77. Kleesiek K, Schmidt M, Gotting C et al. The 536C → T transition in the human tissue factor pathway inhibitor (TFPI) gene is statistically associated with a higher risk for venous thrombosis. *Thromb Haemost*. 1999;82:1–5.
78. Jick H, Slone D, Westerholm B, et al. Venous thromboembolic disease and ABO blood type; a cooperative study. *Lancet*. 1969;1:539–42.
79. Wu O, Bayoumi N, Vickers MA et al. ABO(H) blood groups and vascular disease: a systematic review and meta-analysis. *J Thromb Haemost*. 2008;6:62–69.
80. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol*. 1969;56:111–28.
81. Guba S, Fonseca V, Fink L. Hyperhomocysteinemia and thrombosis. *Semin Thromb Hemost*. 1999;253:291–309.
82. Frosst P, Blom HJ, Milos R et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995;10:111–13.
83. GEHT. Fibrinogen variants: database. site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Database-English-Version/Fibrinogen-variants-Database-_79_.html, accessed date July 28, 2014.
84. de Moerloose P, BoehlenF, Neerman-Arbez M. Fibrinogen and the risk of thrombosis. *Semin Thromb Hemost*. 2010;36:7–17.
85. Ariens RAS. Fibrin(ogen) and thrombotic disease. *J Thromb Haemost*. 2013;11(suppl 1):294–305.
86. Bornikova L, Peyvandi F, Allen G et al. Fibrinogen replacement therapy for congenital fibrinogen deficiency. *J Thromb Haemost*. 2011; 9:1687–704.
87. Haverkate F, Samama M. Familial dysfibrinogenemia and thrombophilia: report on a study of the SCC subcommittee on fibrinogen. *Thromb Haemost*. 1995;73:151–61.
88. Kamphuisen PW, Eikenboom JC, Bertina RM. Elevated factor VIII levels and the risk of thrombosis. *Arterioscler Thromb Vasc Biol*. 2001;21:731–38.
89. Kraaijenhagen RA, Pieternella S, Anker PS et al. High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism. *Thromb Haemost*. 2000;83:5–9.
90. Smith NL, Rice KM, Bovill EG et al. Genetic variation associated with plasma von Willebrand factor levels and the risk of incident venous thrombosis. *Blood*. 2011;117:6007–11.
91. Morange PE, Saut N, Antoni G et al. Impact on venous thrombosis risk of newly discovered gene variants associated with FVII and VWF plasma levels. *J Thromb Haemost*. 2011;9:229–31.
92. Smith NL, Chen MH, Dehghan A et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor. The CHARGE (Cohorts for Heart and Aging Research in genome Epidemiology) Consortium. *Circulation*. 2010;121:1382–392.
93. Meade TW, Mellows S, Brozovic M et al. Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. *Lancet*. 1986;2:533–37.
94. Tripodi A. Levels of coagulation factors and venous thromboembolism. *Haematologica*. 2003;88:705–11.
95. van Hylckama VA, van der Linden IK, Bertina RM et al. High levels of factor IX increase the risk of venous thrombosis. *Blood*. 2000;95:3678–682.
96. Meijers JC, Tekelenburg WL, Bouma BN et al. High levels of coagulation factor XI as a risk factor for venous thrombosis. *N Engl J Med*. 2000;342:696–701.
97. Simioni P, Tormene D, Tognin G et al. X-linked thrombophilia with a mutant factor IX (factor IX Padua). *N Engl J Med*. 2009;361:1671–675.
98. Miyakawi Y, Suzuki A, Fujiti J et al. Thrombosis from a prothrombin mutation conveying antithrombin resistance. *N Engl J Med*. 2012; 366:2390–396.
99. Renné T. The factor-XII-driven plasma contact system. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:242–53.
100. Seligsohn U, Griffin JH. Hereditary thrombophilia. In: Lichtman MA, Beutler E, Kipps TJ et al., eds. *Williams Hematology*, 7th ed. New York: McGraw-Hill; 2006:1981–2007.
101. Mutch NJ, Booth NA. Plasminogen activation and regulation of fibrinolysis. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:314–33.
102. Bolan CD, Krishnamurti C, Tang DB et al. Association of protein S deficiency with thrombosis in a kindred with elevated levels of plasminogen activator inhibitor-1. *Ann Intern Med*. 1993;119:779–85.
103. Zoller B, Dahlback B. Protein S deficiency in a large family with thrombophilia previously characterized as having an inherited fibrinolytic defect. *Thromb Haemost*. 1993;69:1256. Abstract.
104. Wiman B. The fibrinolytic enzyme system: basic principles and links to venous and arterial thrombosis. *Hematol Oncol Clin North Am*. 2000;14:325–38.
105. Fareed J, Hoppensteadt D, Jeske W et al. Acquired defects of fibrinolysis associated with thrombosis. *Semin Thromb Hemost*. 1999;25:367–74.
106. Rand JH, Woolgast LR. The antiphospholipid syndrome. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1216–231.
107. Moore JE, Mohr CF. Biologically false positive serological tests for syphilis: type, incidence, and cause. *J Am Med Assoc*. 1952;150:467–73.
108. Conley CL, Hartmann RC. A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosis. *J Clin Invest*. 1952;31:621.
109. Pengo V, Tripodi A, Reber G et al. Update of the guidelines for measuring the presence of lupus anticoagulants. *J Thromb Haemost*. 2009; 7:1737–740.
110. Warkentin TE, Chong BH, Greinacher A. Heparin-induced thrombocytopenia: towards consensus. *Thromb Haemost*. 1998;79:1–7.
111. Warkentin TE. Heparin-induced thrombocytopenia: a ten-year retrospective. *Annu Rev Med*. 1999;50:129–47.
112. Warkentin TE, Greinacher A. Heparin-induced thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1292–307.
113. Moschowitz E. Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease. *Proc N Y Pathol Soc*. 1924;24:21–24.
114. Amorosi EL, Ultmann JE. Thrombotic thrombocytopenic purpura: report of 16 cases and review of the literature. *Medicine*. 1966; 45:139–60.
115. George JN. The thrombotic thrombocytopenic purpura and hemolytic uremic syndromes. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1292–307.

116. Moake JL, Rudy CK, Troll JH et al. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Eng J Med*. 1982;307:1432–435.
117. Furlan M, Robles R, Lammle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood*. 1996; 878:4223–234.
118. Levy GG, Nichols WC, Lian EC et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. 2001;413:488–94.
119. Sadler JE, Poncz M. Antibody-mediated thrombotic disorders: idiopathic thrombotic thrombocytopenic purpura and heparin-induced thrombocytopenia. In: Lichtman MA, Beutler E, Kipps T et al, eds. *Williams Hematology*, 7th ed. New York: McGraw-Hill; 2006:2031–54.
120. Falanga A, Lee AYY, Rickles FR. Cancer-related thrombosis. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1468–480.
121. Samama MM, Wallenga JM, Kalodiki E et al. Pharmacology and mode of action of heparin and glycosaminoglycans. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1258–266.
122. Shah N, Ortel TL, Schulman S. Vitamin K antagonists: biochemistry, pharmacology, and management. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1267–280.
123. Weitz JI, Gross PL. New antithrombotic drugs. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1318–336.
124. Marder VJ, Stewart DB. Foundations of thrombolytic therapy. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1349—360.
125. Patrono C. Antiplatelet therapy. In: Marder VJ, Aird WC, Bennett JS et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1337–348.



36 Hemostasis: Laboratory Testing and Instrumentation

DAVID McGLASSON, MS
SANDRA ESTELLE, BS
CAROL HILLMAN-WISEMAN, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe necessary precautions regarding specimen collection and processing for coagulation studies, and determine specimen appropriateness.
2. State the principle and clinical significance of each test: platelet function testing (PFT), prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen assay, factor assays, D-dimer assay, and mixing studies.
3. Describe procedures for determining PFT, PT, APTT, TT, fibrinogen assay, and D-dimer assay.
4. Identify the appropriate laboratory procedure for monitoring heparin therapy and oral anticoagulant therapy.
5. Describe the international normalized ratio (INR).
6. Calculate the INR given a patient's PT, mean normal PT, and international sensitivity index (ISI).
7. Interpret the results of routine coagulation testing (i.e., PT, APTT, fibrinogen assay, TT, D-dimer assay, mixing studies).
8. Explain the purpose of point-of-care hemostasis testing.
9. Explain methods of clot detection for each type of hemostasis analyzer described and give the advantages and disadvantages of each method.
10. Distinguish the characteristics of manual, semiautomated, and automated hemostasis analyzers.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Correlate specimen collection, processing, storage, and handling procedures with potential problems in coagulation testing.
2. Defend the use of 3.2% sodium citrate as an anticoagulant for coagulation studies.
3. State the principle and determine the appropriate utilization for each of the following tests: platelet aggregation studies, reptilase time,

(continued)

Chapter Outline

Objectives—Level II *(continued)*

prekallikrein screening test, FXIII screening test, von Willebrand factor (VWF) activity assay, (VWF) antigen immunoassay, dilute Russell's viper venom time, lupus anticoagulants, FVIII inhibitor assay, antithrombin (AT), protein C (PC), protein S (PS), plasminogen, antiplasmin, activated protein C resistance (APCR), and FXa inhibition.

4. Describe the procedure for each test listed in Objective 3.
5. Interpret the results and explain the clinical significance of each test listed in Objective 3.
6. Interpret a set of laboratory results, and suggest the appropriate follow-up or reflex test.
7. Select and defend the most appropriate laboratory tests to monitor anticoagulant therapy, interpret the results, and assess conditions that could affect these tests.
8. Project the potential use of molecular markers in the investigation of a hemostatic problem.
9. Describe testing methodologies that are routinely available on hemostasis analyzers.
10. Identify testing applications employed by various hemostasis analyzers.
11. Identify key performance characteristics that should be evaluated when objectively evaluating the most appropriate coagulation analyzer for an individual laboratory setting.
12. Explain the purpose of incorporating platelet function testing analyzers into the routine hemostasis laboratory.

Key Terms

Agonist
Chromogenic assay
Dilute Russell viper venom time (dRVVT)
Enzyme-linked immunosorbent assay (ELISA)
Global testing
Hypercoagulable state
International normalized ratio (INR)
International sensitivity index (ISI)
Nephelometry
Nomogram
Oral anticoagulant therapy
Pharmacokinetics
Point-of-care (POC) instrument
Pooled normal plasma
Reference interval (RI)
Ristocetin
Ristocetin cofactor (RCoF) assay
Therapeutic range
Turbidometric clot detection
von Willebrand Factor Activity (VWF:A)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the vascular contributions to hemostasis. (Chapter 31)
- Summarize the steps involved in primary hemostasis. (Chapter 31)
- List the coagulation factors and the sequence of events involved in secondary hemostasis. (Chapter 32)
- Describe the biochemical inhibitors that regulate the coagulation cascade. (Chapter 32)
- List the factors and the sequence of events involved in fibrinolysis. (Chapter 32)

Level II

- Define the defect and identify the cause of impaired hemostasis in the following disorders of primary hemostasis: Bernard-Soulier syndrome, Glanzmann's thrombasthenia, drug-induced platelet disorders, and immune thrombocytopenia. (Chapter 33)
- Define the defect and identify the cause of impaired hemostasis in the following inherited disorders of secondary hemostasis: von Willebrand disease, hemophilia A, and hemophilia B. (Chapter 34)
- Summarize the etiology and the pathophysiology of the acquired disorders of secondary hemostasis including disseminated intravascular coagulation, vitamin K deficiency, lupus anticoagulant (antiphospholipid antibody), and FVIII inhibitor. (Chapter 34)
- Summarize the effect of factor V Leiden, protein C deficiency, antithrombin deficiency, and protein S deficiency on the hemostatic system. (Chapter 35)

OVERVIEW

This chapter describes hemostasis laboratory tests and instrumentation used to investigate bleeding and clotting disorders. Patient information obtained during the detailed history and physical examination can assist in determining the coagulation tests needed to diagnose, prevent, or manage a hemostatic abnormality. Screening tests are used to place the defect in one of several broad categories. Special coagulation testing could be necessary to establish a definitive diagnosis. Screening tests for defects of primary hemostasis include a platelet count and/or platelet function testing. Secondary hemostasis screening tests are the prothrombin time (PT), the activated partial thromboplastin time (APTT), fibrinogen, and thrombin time. Screening tests can also be helpful to assess the patient's hemostatic status before surgery.

Reliable coagulation test results depend on the specimen quality. Therefore, this chapter begins with a discussion of specimen collection, handling, processing, and storage. Next, hemostasis laboratory testing in the following areas are discussed: primary hemostasis, secondary hemostasis, and fibrinolysis. A discussion of laboratory tests used to assess hypercoagulable states or thrombophilia and monitor anticoagulant therapy follows. Each test mentioned includes details of the theory, methodologies, procedural comments, and reference values. The technology and principle of the automated instruments utilized in the coagulation laboratory are described. Procedures for selected tests can be downloaded from the chapter's Companion Resources for use in the laboratory component of a hemostasis class. This site also contains additional hemostasis testing information and refers the reader to free information.

INTRODUCTION

The clinical laboratory professional is an integral part of the medical team involved in the diagnosis of bleeding and clotting disorders. Extensive knowledge of the physiology of hemostasis and coagulopathies (Chapters 31–35) is needed to understand the testing for the coagulopathies and provide the best care possible for the patient. Diagnosis of a bleeding problem generally utilizes an algorithmic approach. When laboratory professionals are knowledgeable of the testing sequence, they can assist the clinician with specimen collection (e.g., minimize frequency and amount of blood drawn), reflex testing, and prioritizing testing. The algorithmic approaches for thrombophilia and bleeding problems allow the clinical laboratory professional to follow the progression of screening tests and to select the specific tests for follow-up testing.

In addition to describing testing for the adult population, this chapter provides additional information for the pediatric population, which includes the newborn, infant, toddler, and children to age 17 years. Laboratories must be cognizant of the need for smaller sample size (blood sampling and testing) for this group of patients. Because congenital deficiencies are more likely to be discovered and diagnosed in the pediatric age group, tests must be sensitive to the very low levels of the constituent being measured.

The chapter emphasizes the technical information concerning the blood sample(s) needed for the particular test(s). A common phrase regarding coagulation is "the result is only as good as the sample." Therefore, the laboratory professional must have a good understanding of specimen collection, handling, processing, and storage. If the final result is questionable or illogical, specimen integrity should be questioned. The coagulation laboratory should establish its own normal range or reference interval for each analyte. The reference interval provided by the commercial company or a published source can be used as a temporary reference. Reference intervals are established by testing age-appropriate, normal (healthy) donors (an equal number of males and females) using the laboratory's own instrument/reagent combination. The normal donors must be questioned to rule out a history of bleeding or thrombotic disorders, acute infections, pregnancy, liver or renal disease, and postsurgical bleeding (e.g., dental extractions). A normal donor must also be excluded if the medication history includes any of the following: oral contraceptives, hormone replacement therapy, or herbal supplements. Unacceptable sources for normal donor blood include pre-employment individuals and presurgery patients. Reference interval testing should be performed over a minimum of a 5-day period to factor in the intralaboratory day-to-day variability. If normal donor blood samples are not available, commercial companies can provide frozen citrated plasma samples from individuals who have been prescreened.

Specialized testing can include functional and antigenic measurement of the analyte being evaluated. For available functional assays, the theory is described. It is essential to know whether the analyte measured is functioning normally. The antigenic assay is performed to determine whether the protein concentration is abnormal. Occasionally, the antigenic assay is normal but the functional assay is abnormal. Functional assays are available for most analytes. Most algorithms are designed to perform the functional assay first, and if abnormal, the antigenic assay can be requested.

Some tests described in this chapter might be available only at a reference laboratory. The laboratory professionals and patient care providers must follow the same quality control guidelines for specimen collection, handling, processing, and storage of the samples that are sent to the reference laboratory.

The Companion Resources include a wealth of technical information for the medical laboratory professionals working in a coagulation laboratory. The information is practical in nature and is included to improve coagulation testing. The figures and tables can assist in modifying existing techniques and instituting new protocols (e.g., calculating FVIII inhibitors, heparin nomograms, commercial sources of normal and abnormal plasmas for reference ranges).

SPECIMEN COLLECTION AND PROCESSING

The accuracy of coagulation testing relies on properly collecting, processing, and storing the specimen (Web Table 36-1).

Specimen Collection

A two-syringe or two-tube technique formerly was recommended when collecting blood with a syringe or an evacuated tube system. The blood in the first syringe or tube was discarded to minimize contamination with tissue factor, and the second syringe or tube was used for the coagulation specimen.[1] The current Clinical and Laboratory Standards Institute (CLSI; 2008:H21-A5) guidelines do not require a discard tube for PT and APTT testing.[2] Blood collection tubes used

for coagulation testing should not be used after collection tubes with additives, such as heparin or ethylenediaminetetracetic acid (EDTA), to prevent carryover and contamination into the coagulation sample.

Blood collection by direct venipuncture is preferred for coagulation testing. Butterfly needles of 21–22 gauge with polystyrene tubing attached to a syringe or vacutainer system are used for adult and pediatric patients with difficult venous access. A needle with a diameter smaller than 0.7 mm (larger than a 22-gauge needle) could prolong blood collection and because of the high pressure gradient in the needle could cause hemolysis and platelet activation.[3]

If butterfly needles are used, the two-tube technique alleviates the problem of underfilling the citrate tube and causing an error in the blood to anticoagulant ratio. The air in the tubing displaces an equivalent volume of blood in the first tube, causing it to inadequately fill. No air is in the tubing when the second tube is drawn, so this tube fills to the proper level. A complete fill is essential to achieve the proper ratio of blood to anticoagulant in the citrate tube (9:1 blood-to-anticoagulant ratio).

Drawing blood through an indwelling catheter requires that care be taken to avoid heparin contamination of the sample. Heparin is used to keep the catheter line free flowing. To prevent contamination, the catheter line should be flushed with saline, and the first 5 mL (less volume in pediatric patients) of blood should be discarded. If the laboratory suspects heparin contamination of the sample, a commercial heparinase product can be added to the plasma to neutralize the heparin. Up to 2 USP units of unfractionated heparin in 1 mL of citrated plasma can be neutralized and the sample retested.[4] Heparin neutralization should not be performed when the sample collected is for heparin therapy monitoring. In this case, a sample must be recollected from a non-heparinized site to ensure accurate test results.

The anticoagulant of choice for coagulation studies is 3.2% buffered sodium citrate (105–109 mmol/L). The recommended ratio for coagulation testing in sodium citrate is nine parts whole blood and one part anticoagulant (9:1). The 3.2% sodium citrate concentration, as opposed to the 3.8% concentration formerly recommended for coagulation studies, alleviates the problems associated with excess citrate in samples that have high hematocrits.[5] CLSI guidelines, however, still recommend adjusting the amount of anticoagulant for the 3.2% concentration when the patient's hematocrit is >55%[2] (Figure 36-1 ■).At this concentration, the plasma volume is lower. The excess free citrate binds a higher proportion of calcium ions that are required for calcium-dependent clotting assays. This could lead to falsely prolonged clotting times (CTs), especially affecting the PT and APTT. Evacuated citrate tubes that draw volumes of 4.5, 2.7, and 1.8 mL are available. If the citrate tube is overfilled, insufficient calcium can be bound, and clotting can occur in the tube (consuming some of the clotting factors), producing falsely prolonged results similar to a consumptive coagulopathy.

Accurate labeling of the citrated blood samples is critical. The sample must be labeled with the appropriate identifiers according to the institutional and regulatory agencies guidelines in the patient's presence. Additional information for further identification of the sample (i.e., before or after infusion, time of draw) should also be included to eliminate sample and testing errors. Coagulation testing can be used to calculate a response to the administration of a specific factor

$$\mathbf{C = (1.85 \times 10^{-3}) \cdot (100 - Hct) \cdot V}$$

C = Volume of sodium citrate
V = Volume of whole blood drawn
Hct = Patient hematocrit

For example, to draw 5 mL of blood from a patient with a hematocrit of 63% you calculate the amount of sodium citrate to use as follows:

$$(1.85 \times 10^{-3}) \cdot (100 - 63) \cdot 5\text{mL} = 0.34\text{ mL}$$

So mix 5 mL of blood with 0.34 mL of sodium citrate

■ **FIGURE 36-1** When blood is drawn for coagulation testing in a patient with a hematocrit >55% using the 1:9 ratio of buffered sodium citrate to blood, the plasma contains excess citrate that can affect coagulation testing. A correction formula to adjust the citrate:blood ratio is recommended in these cases. The calculation used is presented here.

concentrate, establish the baseline level of a factor, or monitor anticoagulation therapy, all of which require precise sample identification.

The interactions of the laboratory staff with the health care team are important for obtaining a sample at the proper time. The patient's history of receiving blood products should be checked because testing done within the half-life of administered clotting factor or platelet products could measure the transfused component as well as the patient's own component. Some dietary supplements and drugs can interact and affect hemostasis (Web Table 36-2).[6] Certain fibrinolytic factors have diurnal variability; therefore, each laboratory needs guidelines on when to draw these samples. Before performing platelet function testing, the ordering physician and nursing or laboratory staff must thoroughly question patients regarding medications because some affect the testing for the life of the platelet (7–10 days).

Specimen Processing

Hemostasis testing is performed predominately on platelet-poor, citrated plasma samples. Platelet-rich plasma and whole blood may be used for platelet function testing. Whole blood also is used for global coagulation testing.

Platelet-Poor Plasma (PPP)

To obtain PPP, the capped citrated specimen is centrifuged at room temperature for at least 15 minutes at 1500–2500 g. Each lab must adjust the speed and time of centrifugation in order to achieve a PPP sample with a residual platelet count of less than 10,000/mcL (μL). Coagulation testing can be performed by sampling the PPP directly from the centrifuged tube, or the PPP can be removed from the tube for later testing or storage. When removing the PPP, a small interface of PPP should be left over the packed cell layer. This ensures that the platelet layer is undisturbed and that platelets have not been resuspended into the PPP.

The use of PPP is essential for technical reasons. Platelets contain platelet factor 4 (PF4), which neutralizes heparin (thus affecting sample's testing for the presence of heparin). Platelets contain phospholipids, which affect lupus anticoagulant testing and factor assay testing (especially if the sample is frozen and thawed). Platelets also contain proteases, which, when released during the thawing of a frozen sample, can alter results for von Willebrand factor testing.

A clot of any size in the sample renders it unacceptable. The whole blood specimen should be checked visually for clots while *gently* rotating the capped sample end to end several times. The specimen also can be checked for fibrin or clots after removal of the plasma or when testing is completed. After removing the plasma, wooden applicator sticks can be twirled inside the tube and pulled out to check for fibrin threads or clots. Coagulation testing is most reliable when performed on a fresh sample. If testing is delayed, the PPP can be stored at 18–24°C or 2–8°C for up to 4 hours before performing most tests. If the testing cannot be completed within 4 hours, the PPP can be stored at −70°C for 6 months to 1 year. Frozen samples must be thawed rapidly at 37°C because excessive heating (>5 minutes) can result in the loss of the labile factors V and VIII. Samples for coagulation testing should never be stored in self-defrosting freezers. The freeze–thaw cycles could rupture any residual platelets in the plasma, activate coagulation factors, and compromise sample integrity and stability.

Platelet-Rich Plasma (PRP)

PRP is obtained to perform studies of platelet function (light transmittance platelet aggregometry [LTA]). Low centrifugation spins that yield PRP at a concentration of 200,000/mcL must be determined for LTA testing.

Citrated Whole Blood

When testing requires citrated whole blood, the sample is drawn as previously described. Whole blood samples should be mixed after collection and before use by gently rotating the tube end to end 4 or 5 times. This sample type can be used for platelet function testing (i.e., platelet function analyzer [PFA], whole blood aggregation, or one of the global coagulation tests, such as thromboelastography [TEG]). Because platelets can be activated by foreign surfaces, the sample should not be checked for a clot with the wooden applicators before testing has been completed.

☑ CHECKPOINT 36-1

Why have most clinical laboratories switched from 3.8% to 3.2% sodium citrate for specimen collection in coagulation studies?

LABORATORY INVESTIGATION OF PRIMARY HEMOSTASIS

Laboratory testing to evaluate primary hemostasis includes tests for platelet concentration and function. Clinical laboratory professionals must be aware that a wide variety of drugs can affect platelet function for the life span of the platelet (7–10 days).[6]

Screening tests for platelet function include the bleeding time (BT) and in vitro testing using the PFA. However, many investigators consider the BT an obsolete procedure.[7–9] Definitive testing for platelet function includes PRP and/or whole blood platelet aggregation. More specialized platelet function testing can be performed by flow cytometry. There are also tests available to evaluate acquired states of thrombocytopenia such as heparin-induced thrombocytopenia (HIT) and neonatal alloimmune thrombocytopenia (NAIT) (Chapters 33 and 35).

Bleeding Time

The BT is an in vivo measurement of platelet function. In addition, it is affected by platelet number and vascular integrity (Chapter 31). The BT measures the time required for bleeding to cease from a superficial skin incision.

The general **reference interval (RI)** for the BT is 1–9 minutes.[10,11] Patients with platelet counts less than 100×10^9/L usually have a prolonged BT. The BT has been discredited in recent years as being unreliable as a screening test for surgical patients. It lacks reproducibility and is affected by location of the incision, pressure applied when performing the incision, operator experience, and patient factors such as age, gender, diet, hematocrit, skin elasticity, medications, and platelet count.[7–9]

In the absence of a clinical history of a bleeding disorder, the BT is not a useful predictor of the risk of hemorrhage associated with surgical procedures (i.e., a normal BT does not exclude the possibility of excessive hemorrhage). Many hospitals have stopped using the BT.[7–9]

Platelet Function Analyzers

There is point-of-care (POC) and more highly complex instrumentation for evaluating platelet function. Following is a description of these instruments and their use in assessing platelet function.

PFA-100®, 200®; Siemens Healthcare, Inc.

The PFA in vitro method for assessing and screening platelet function is a more standardized technique, eliminating the variables discussed with the BT (Web Figure 36-1). The assay measures platelet function in whole blood at a high shear rate. Citrated blood is added to each of three reservoirs, one with collagen/epinephrine, a second with collagen/adenosine diphosphate (ADP), and a third with a low concentration of ADP and prostaglandin E1 (PGE1). The instrument aspirates whole blood through a capillary with a 150 mcM (μM) aperture with a bioactive membrane coated with collagen/epinephrine, collagen/ADP, or ADP/PGE1. A pressure sensor detects the formation of a platelet plug. The time to occlude the aperture (the "closure time") is a function of platelet count, platelet activity, von Willebrand factor (VWF) activity, and hematocrit. This method is sensitive to von Willebrand disease (VWD), aspirin-induced platelet dysfunction, clopidogrel interference of platelet aggregation, and aggregation defects (all resulting in prolonged or abnormal closure times). A reference interval is established for each reservoir using a standardized blood-drawing technique (size of needle, citrate concentration).[11–15] Refer to Table 36-1 ★ for expected results with the PFA.

VerifyNow Accumetrics Assay System (San Diego, CA)

This is a POC device that measures platelet-induced aggregation. It is a turbidimetric optical detection system that measures aggregation by LTA. It uses a designated cartridge that employs a microbead agglutination technology for assaying antiplatelet medications such as aspirin, clopidogrel, and integrilin. Each cartridge contains a lyophilized

★ **TABLE 36-1** Expected Results with the PFA

Condition	PGE1/ADP	COL/EPI	COL/ADP
Normal	Normal	Normal	Normal
Acetylsalicylic acid (ASA)	Normal	Abnormal	Normal
VWD	Unknown	Abnormal	Abnormal
Glanzmann's thrombasthenia	Abnormal	Abnormal	Abnormal
Clopidogrel	Abnormal	Variable; not recommended	Variable; not recommended

preparation of human fibrinogen-coated beads with arachidonic acid (AA) as an agonist for aspirin testing and ADP and PGE1 for testing for the antiplatelet effects of clopidogrel. The integrilin device uses human fibrinogen-coated beads with a thrombin receptor (protease-activated receptor-1)–activating peptide as the **agonist** (aggregating reagent). The system uses a 3.2% sodium citrated 2.0 mL collection tube. An aliquot of the specimen is dispensed from the tube to the assay cartridge, and results are generated in set units for each cartridge. Each device has its own manufacturer-suggested ranges for the cutoffs to denote the effects of the antiplatelet medications.[16,17]

Platelet Aggregometry

Platelet aggregation studies are comprehensive studies of platelet function. Blood is collected in 3.2% sodium citrate. The whole blood can be used for testing (measuring luminescence and impedance), or PRP can be prepared from the sample for optical platelet aggregometry.

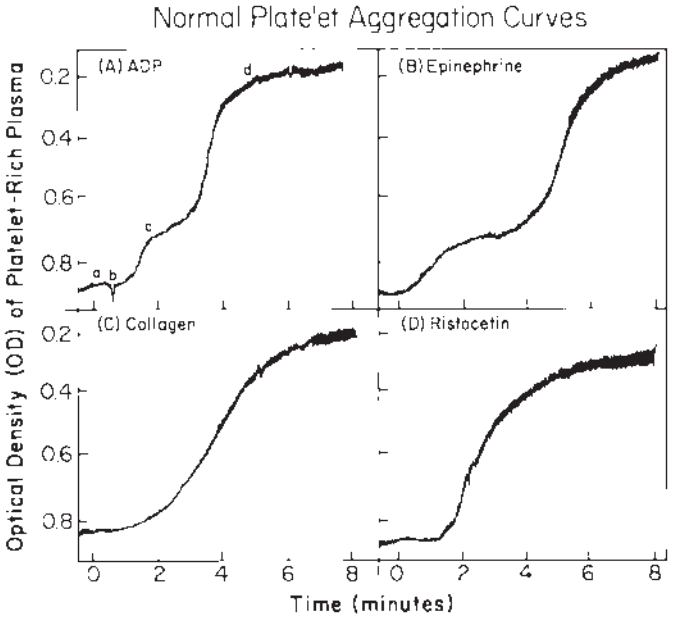
Platelet-Rich Plasma (PRP) Aggregation

PRP is carefully prepared from a citrated blood sample by adjusting the platelet count with the patient's own PPP to a standard number (usually 200,000/mcL). However, some investigators do not recommend the adjustment procedure because it further manipulates the platelets and can cause spontaneous aggregation.[13] The sample is stirred, warmed to 37°C in a special photometric device (an aggregometer), and an agonist is added. In the presence of the agonist, the platelets begin to aggregate, resulting in a change in optical density (OD) of the PRP that the aggregometer records as a graph (curve). Commonly used platelet agonists include ADP, epinephrine, collagen, ristocetin, and arachidonic acid.

Depending on the agonist used and its concentration, a primary and/or secondary wave of aggregation can occur (see Figure 36-2 ■). The primary wave reflects the direct response of the platelets to the aggregating reagent and represents platelet shape change and the formation of small aggregates. The secondary wave represents the complete aggregation response, which occurs as a result of endogenous ADP being released from the activated platelet dense bodies. An aggregating reagent that results in both a primary and secondary wave is said to produce a *biphasic curve*, whereas an aggregating reagent that results in only one wave is said to produce a *monophasic curve*. Changes in platelet aggregation curves are interpreted to identify qualitative platelet disorders (Table 36-2; Chapter 33). Aggregating reagents produce typical aggregation patterns.[18]

Ristocetin is unique among the agonists in that its action depends on the interaction of plasma VWF and the platelet membrane glycoprotein GPIb/IX/V receptor and technically represents platelet agglutination rather than aggregation (Chapter 31). Ristocetin-induced platelet agglutination (RIPA) can be abnormal in both VWD and Bernard-Soulier syndrome (BSS; a defect of GPIb/IX/V), whereas plasma ristocetin cofactor activity (RCoF or VWF:A, discussed in "von Willebrand Factor Activity (VWF:A)") is usually decreased in VWD but is normal in BSS. In addition, the RIPA defect observed in VWD can be corrected by adding normal plasma to the patient's PRP because the defect is a deficiency of a plasma protein, not of the platelets. The RIPA agglutination defect in BSS is not corrected with the addition of normal plasma because it is the result of a deficiency of the GPIb/IX complex on the patient's platelet membrane.

The effects of aspirin or aspirin-containing drugs on platelet function can be evaluated with the use of AA (Web Table 36-3). Aspirin irreversibly inhibits platelet function for 7–10 days (the life



■ **FIGURE 36-2** Normal platelet aggregation curves. Normal responses to the commonly used aggregating reagents: ADP, epinephrine, collagen, ristocetin. **(a)** Baseline before addition of the reagents. **(b)** Initial increase in absorbance that occurs immediately following the addition of reagent, representing a change in platelet shape. **(c)** The primary wave of aggregation. **(d)** Secondary wave of aggregation.

span of the platelet). If a secondary wave of aggregation is not produced with ADP or epinephrine, using AA can help to differentiate an acquired (drug) defect from an inherent platelet defect. Drugs such as the thiopyridines (e.g., clopidogrel, prasugrel) and other medications such as cangrelor and ticagrelor affect the ADP receptors of the platelet and block platelet aggregation using that agonist. Platelet release defects or storage pool disease (Chapter 33) produce aggregation results with ADP and epinephrine similar to those seen with aspirin ingestion but can be differentiated by using AA (Table 36-2 ★, Figure 36-3 ■).

Decreasing concentrations of epinephrine, which produce aggregation of patient PRP but not normal control PRP (Web Figure 36-2), have been used to diagnose "sticky platelet syndrome," a term adopted by E. F. Mammen to describe patients who had platelet hyper-responsiveness to low doses of ADP and/or epinephrine.[19] These patients often had unexplained arterial vascular occlusions. The precise etiology of this syndrome is not known, but abnormal platelet receptors maybe involved. Normal plasma levels of PF4 and β-thromboglobulin (βTG) suggest that the platelets are not activated at all times. Platelet aggregation using decreasing concentrations of epinephrine is not sensitive for demonstrating sticky platelet syndrome in pediatric populations.[20]

Some aggregometers can measure adenosine triphosphate (ATP) secreted from dense granules, either by whole blood or PRP-optical methodologies. Luminescence assays improve the diagnosis of storage pool disease because an actual amount of ATP, directly proportional to the dense granule content, is measured. Measuring the amount of ATP is more accurate than evaluating whether the platelet aggregation curve is biphasic.[13,21–23]

Whole Blood Aggregation

The use of whole blood aggregation allows for a smaller sample size than is used in PRP aggregation and is a quicker analytic method because PRP does not have to be prepared. However, there is no standardization of the platelet count. The whole blood assay measures electrical resistance across two metal wires (probes). The whole blood sample is initially exposed to a small electric current that coats wires with a monolayer of platelets. Upon the addition of an agonist, platelets form aggregates on the monolayer, adding electrical resistance (ohms) to the circuit. The change in impedance is measured as a function of time. The citrated sample is challenged with ADP, collagen, AA, and thrombin. The use of the agonist thrombin allows for determining the content of the dense granules. Thrombin causes dense granules to release ATP, so whole blood aggregation with thrombin can efficiently assess whether the patient has δ-storage pool disease. With certain instruments, the sample can also be evaluated for luminescence, as was done with PRP.[13]

Multiplate® Platelet Function Analyzer

The Multiplate® Platelet Function Analyzer (Roche, Indianapolis, Indiana, distributed by Diapharma, Inc., West Chester, Ohio) is a new platelet aggregometer that uses whole blood. The Multiplate system detects the effects of antiplatelet inhibitors such as aspirin, clopidogrel, and GPIIb/IIIa antagonists. It uses a small amount of blood for each test (0.3 or 0.175 mL). The instrument looks at three platelet aggregation parameters (the velocity of aggregation, maximum aggregation, and the area under the curve [AUC]), which is the most important. The total height of the aggregation curve and the slope of the aggregation response affect AUC . The AUC seems to

★ **TABLE 36-2** Typical Pattern of Response to Aggregating Reagents in Qualitative Platelet Disorders[a]

Qualitative Platelet Disorders	Aggregating Reagents				
	Collagen	ADP	Epinephrine	Ristocetin	Arachidonic Acid
Normal	Monophasic curve (secondary wave only): representing secondary platelet aggregation associated with release of endogenous ADP	Biphasic curve with 2×10^{-5} M ADP: demonstrating primary and secondary waves of platelet aggregation	Biphasic curve: demonstrating primary and secondary waves of platelet aggregation	Biphasic curve: demonstrating primary wave resulting from immediate platelet agglutination; secondary wave associated with platelet release induced by platelet agglutination	Monophasic curve (secondary wave only): representing effect of thromboxane A_2 on secondary platelet aggregation
von Willebrand disease	Normal	Normal	Normal	No response	[b]
Bernard-Soulier syndrome	Normal	Normal	Normal	No response	[b]
Glanzmann's thrombasthenia	No response	No response	No response	Normal	[b]
Storage pool disease	No response	Primary wave only	Primary wave only	Normal	Normal
Aspirin ingestion	No response	Primary wave only	Primary wave only	Normal	Suppressed response
Clopidogrel ingestion	Normal	No response	Primary wave only	Normal	Normal

[a]Chapter 33.

[b]Not applicable. Arachidonic acid evaluates the thromboxane A_2 synthetic pathway.

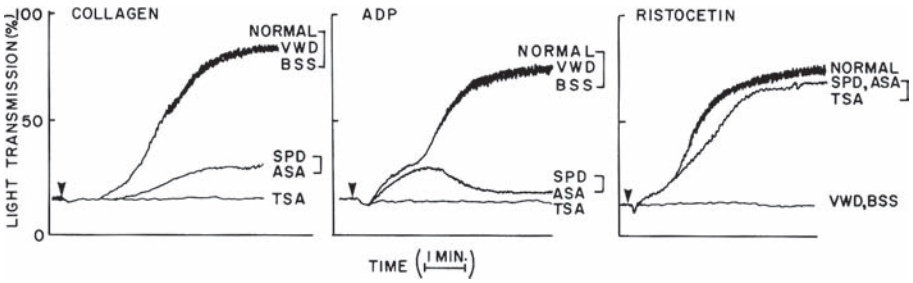


FIGURE 36-3 Platelet aggregation patterns in various disorders. Typical tracings obtained using concentrations of collagen, ADP, and ristocetin illustrate the differences between platelet aggregation in normal subjects and that in patients with VWD, BSS, TSA, SPD, and aspirin ingestion and ASAs. Impaired release of ADP accounts for the SPD and ASA defects. VWF corrects the defective ristocetin aggregation in VWD but not in BSS.

ADP = adenosine diphosphate; VWD = von Willebrand's disease; BSS = Bernard-Soulier syndrome; TSA = thrombasthenia; SPD = storage pool disease; ASA = aspirin-like disorders; VWF = von Willebrand's factor

be the best for overall assessment of platelet activity. Testing is performed using a disposable cuvette that simultaneously performs the assay with two independent sensors in the test cuvette. The results are calculated by the software and are expressed as mean values of the parameters determined with each sensor. The correlation coefficient (cc) between the values of the two individual curves is determined and is deemed accepted when the cc is ≥0.98. If the results are not within a specified range the operator is alerted. All of the results are in laboratory-determined units set by in-house testing. Abnormal results could indicate that the antiplatelet treatment plans for patients need to be modified.[23–25]

Additional Tests Evaluating Platelets

Flow Cytometry

New approaches for laboratory diagnosis of the platelet disorders BSS (GPIb/IX deficiency) and Glanzmann's thrombasthenia (GPIIb/IIIa deficiency) involve flow cytometry using monoclonal antibodies directed against the platelets' respective glycoproteins. A panel of antibodies that recognize the resting, activated, and ligand-occupied forms of the GPIIb/IIIa complex are available.[25] Thus, both the presence and the functional activity of this complex can be measured. Testing can also be performed to evaluate the presence of circulating activated platelets to aid in diagnosing thrombotic syndromes.[26]

Clot Retraction

The physiologic phenomenon and the screening test of clot retraction, a function of activated platelets, have been described in Chapter 31. This assay is rarely performed in specialty coagulation laboratories.

Tests for Diagnosing Heparin-Induced Thrombocytopenia (HIT)

Platelet destruction through immune mechanisms can cause thrombocytopenia. One of the more common causes is heparin-induced thrombocytopenia. Historically, a functional heparin–platelet aggregation assay using normal platelets and the patient's serum was used to screen for HIT. In the presence of HIT immunoglobulins in the patient's serum, the normal donor PRP platelets were activated when heparin was added. The procedure was time consuming and often resulted in false negative or positive results. The current gold standard for the diagnosis of HIT is a platelet 14 C serotonin release assay whose complexity limits its availability to reference laboratories. An **enzyme-linked immunosorbent assay (ELISA)** for detecting antibodies against heparin/PF4 complexes (Chapter 33) is also available and widely used. The results obtained with the serotonin release assay and ELISA generally are in agreement in patients with a clinical diagnosis of HIT. However the ELISA detects antiheparin antibodies in patients who do not have heparin-induced thrombocytopenia, and therefore can produce a false positive diagnosis if used to diagnose HIT.[27]

☑ CHECKPOINT 36-2

What affect does aspirin taken daily for a heart condition have on a patient's platelet functional studies? Explain.

LABORATORY INVESTIGATION OF SECONDARY HEMOSTASIS

Laboratory tests of secondary hemostasis evaluate coagulation factors and inhibitors. The testing of patients for secondary hemostasis starts with screening tests. An algorithm can provide guidelines for evaluating a bleeding diathesis. The following section includes technical information to help understand the variability within each assay. This chapter's Companion Resources should also be consulted for methodologies, quality control, pediatric considerations, and expanded coverage of the topics.

Screening Tests

Screening tests include the PT, APTT, TT, and quantitative fibrinogen. Abnormalities in any of these tests require further testing. The battery of reflex testing is based on the results of the screening tests.

Prothrombin Time (PT)

The PT is an important screening test for the laboratory evaluation of patients with inherited or acquired deficiencies in what previously was known as the *extrinsic pathway* and is now referred to by some as the *extrinsic X-ase pathway* (pronounced as "ten-ase") of the coagulation cascade. Historically, the PT also has been used to monitor oral anticoagulant therapy (see "Oral Anticoagulant Therapy and the Prothrombin Time—INR Value" in this chapter). In vivo, TF activates the coagulation cascade via the formation of the TF/FVIIa complex.[28] In the PT test, thromboplastin (TF/calcium mixture) is added to citrated PPP, and the time for fibrin formation is measured. Numerous commercial sources of thromboplastins (most commonly rabbit brain and recombinant human tissue factor) are available; they vary in sensitivity to coagulation factor deficiencies. Variabilities result from differences in animal and tissue thromboplastin sources as well as the method of reagent preparation. Each laboratory must establish its own reference interval (RI) or geometric mean PT for its reagent/instrument combination.

The procedure for establishing a specific reagent/instrument RI and technical information is in the text's Companion Resources. Clot formation can be detected by optical or electromechanical automated instrumentation, manual methods, or POC monitoring devices. The general reference interval for the PT is 12–15 seconds. The PT can be prolonged because of deficiency of factors VII, X, V, II (prothrombin), and fibrinogen or the presence of an inhibitor.

Prolongation of the PT is also seen with proteins induced by vitamin k absence or antagonists or antagonism. These proteins are immunologically similar to the naturally occurring factors but are dysfunctional because they lack the γ-carboxyglutamic acid residues required for Ca^{++} and phospholipid binding during clotting.

Activated Partial Thromboplastin Time (APTT)

The APTT is an important screening test for the laboratory evaluation of patients with inherited or acquired deficiencies of proteins in the intrinsic pathway, also known as the *intrinsic X-ase pathway of the coagulation cascade*. The APTT also can be used as a screening test for the detection of circulating inhibitors of blood coagulation (e.g., lupus anticoagulants) (Chapters 32, 34, 35).

The sensitivity of the various commercial APTT reagents to factor deficiencies and to lupus anticoagulants varies greatly. Therefore, each laboratory must evaluate the APTT reagent to be used. The screening tests (PT and APTT) detect a factor deficiency only when the factor decreases to a level of 25–40% of normal (depending on the reagent or factor).[29] The APTT has been the most common procedure used to monitor the effectiveness of standard (unfractionated) heparin therapy. (See "Laboratory Evaluation of Anticoagulant Therapy" in this chapter.) The APTT uses two reagents: an activated partial thromboplastin and Ca^{++}. The partial thromboplastin reagent simulates activated platelet surfaces by providing phospholipids on which enzymatic reactions in the coagulation cascade can occur. In addition the reagent contains an activator (kaolin, celite, micronized silica, or ellagic acid) that provides the negatively charged surface for the activation of FXII and prekallikrein.[30–32] The citrated PPP and APTT reagent are incubated at 37°C for approximately 3 minutes. Activation of the contact factors occurs during the incubation. Next, calcium chloride is added, and the time required for a fibrin clot to form is measured. Details of this procedure are provided in the Companion Resources. Clot formation can be detected by optical or electromechanical automated instrumentation or manual methods. The general reference interval for adults is in the range of 25–35 seconds but varies by laboratory. See Chapter 34, Table 34-14, for reference intervals for preterm and term infants.

The APTT also evaluates prekallikrein (PK) and high-molecular-weight kininogen (HK). Deficiency of FXII, PK, or HK can result in a markedly prolonged APTT in the absence of clinically significant bleeding.

☑ CHECKPOINT 36-3

Routine coagulation testing was performed on a properly collected and processed specimen. The PT result was prolonged, but APTT was normal. What is the best interpretation of these results?

Thrombin Time (TT)

The TT has an important role as a screening test because it measures the conversion of fibrinogen to fibrin by adding excess thrombin to undiluted plasma. Because the other intrinsic and extrinsic clotting factors previously measured in the PT and APTT have no effect on this test, TT is useful for evaluating other parameters affecting the formation of fibrin. Problems with the conversion of fibrinogen to fibrin can occur as the result of hypofibrinogenemia or dysfibrinogenemia (Chapter 34), the presence of heparin or direct thrombin inhibitors, and the presence of fibrin degradation products (FDPs). In rare cases, autoantibodies against bovine thrombin (e.g., induced by topical thrombin application or the use of fibrin sealants) and paraproteins also can interfere with fibrin formation and result in a prolonged TT.[33] The TT is useful to confirm an abnormal FDP result and verification of heparin in a citrated PPP sample. An extremely prolonged TT usually indicates a heparin effect. If the sample is contaminated with heparin, the heparin can be neutralized with Hepzyme. The testing can then be repeated, or the specimen can be redrawn.

In the TT procedure, citrated PPP is incubated at 37°C. Thrombin reagent is added, and the time for a clot to form is measured. The general reference interval for the TT is 15–19 seconds. The TT in preterm and term infants is longer than the adult reference interval even though the fibrinogen level is within the same normal reference interval, which can be explained by the presence of a distinct fetal fibrinogen molecule with altered function. The TT generally becomes normal within a few days after birth.

Quantitative Fibrinogen Assay

Several methods have been used to determine the fibrinogen concentration including turbidimetric precipitation or denaturation methods with some instrumentation calculating a result derived from the PT's slope. The reference method for fibrinogen determination

is the Clauss assay, a clot-based functional measurement in which thrombin is added to various dilutions of known concentrations of fibrinogen (reference plasma or calibrator) to produce a thrombin-CT in seconds. The clotting times are then plotted on a log/log graph (reference curve) with known concentrations on the x-axis versus CT (TT) on the y-axis (Figure 36-4 ■).[34] Thrombin CTs are identified using appropriate controls (normal and abnormal) and the patient PPP at a 1:10 dilution. The fibrinogen concentration is inversely proportional to the CT. If fibrinogen levels are low, more concentrated (i.e., less dilute) dilutions of patient plasma (1:5 or 1:2) are prepared so that fibrinogen can be accurately determined using the reference curve with the result divided by 2 (if a 1:5 dilution) or 5 (if a 1:2 dilution). FDPs less than 100 mg/dL do not affect the assay at the normal 1:10 dilution provided the fibrinogen concentration is 150 mg/dL or higher. If the dilution is lower (i.e., 1:5 or 1:2), however, interference can occur in the procedure.

The fibrinogen results for the controls and the patient are determined from the reference curve using their respective CTs. In general, the reference interval for fibrinogen (adults and the pediatric population) is 200–400 mg/dL (Chapter 34, Table 34-14).

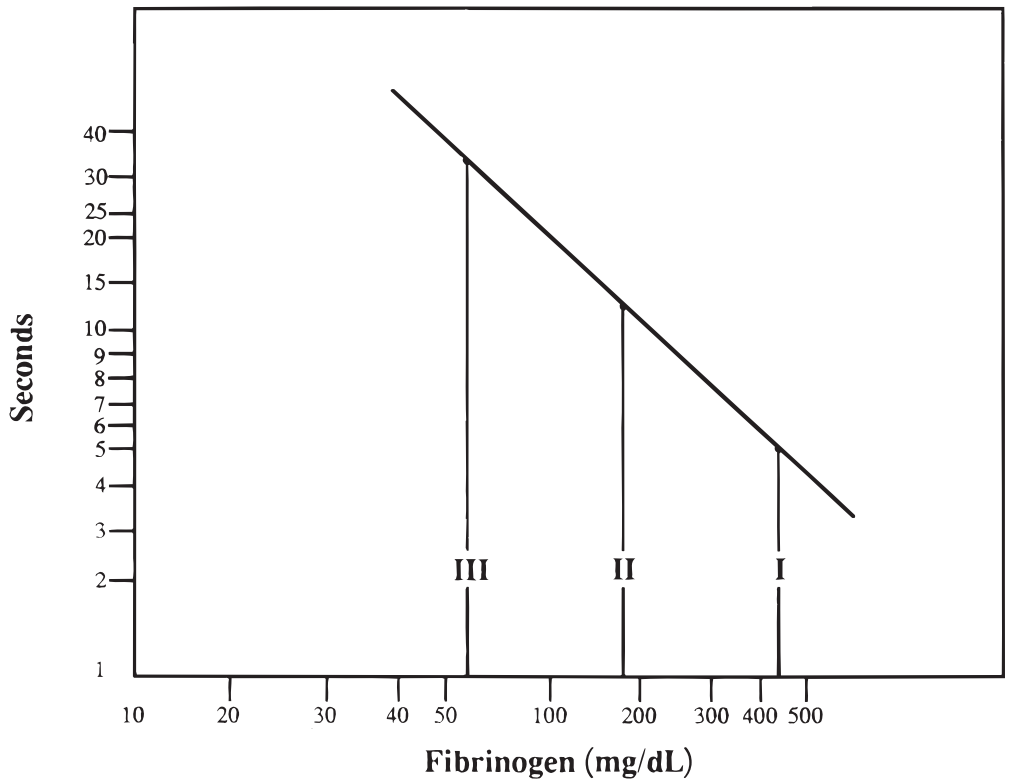
Decreased levels of fibrinogen occur in acquired disorders such as disseminated intravascular coagulation (DIC), primary and secondary fibrino(geno)lysis, liver disease, and congenital disorders such as dysfibrinogenemia and hereditary afibrinogenemia (Chapter 34). When an abnormal fibrinogen is suspected, an antigenic (immunological) test is performed. Increased levels of fibrinogen can occur in inflammatory disorders, cardiovascular disease, pregnancy, and use of oral contraceptives by women because fibrinogen is an acute phase reactant protein.

Tests to Identify a Specific Factor Deficiency

When the PT and/or APTT is/are prolonged, further testing can be performed to identify the specific cause of the abnormality. To assist in selecting the appropriate diagnostic test after the screening tests, an algorithm can be followed. As each of the following specific tests is described, its sensitivity(ies) to decreased levels of clotting factor(s) and whether these levels would be detected in the specific screening test are discussed.

Mixing Studies

Mixing studies (also known as *circulating anticoagulant screen* or *screening test for circulating inhibitor*) are performed to differentiate a factor deficiency from the presence of a circulating inhibitor.[35] These studies repeat the screening tests that have abnormal results (PT, APTT) using several different dilutions of the patient's PPP mixed with a **pooled normal plasma (PNP)** (Web Table 36-4). Because factor levels of at least 50% of normal are generally sufficient to produce a normal PT and APTT result, repeating the PT and APTT with dilutions of patient and PNP corrects the prolonged patient result if it is caused by a deficiency of one or more procoagulant factors.



■ **FIGURE 36-4** Fibrinogen standard curve. I, II, and III represent reference plasmas of known fibrinogen concentrations. The TT in seconds is on the y-axis, and the fibrinogen concentration in mg/dL is on the x-axis.

TT = thrombin time

The testing is performed immediately after mixing patient and PNP and again after incubating the mixture at 37°C.

The CTs for the various dilutions and time intervals are compared to determine whether the patient's prolonged CT has been corrected by the addition of PNP. Clotting times tend to increase with time and incubation because of the loss of labile factors (FV and FVIII); therefore, comparing the patient's diluted sample result with the result obtained from the PNP is important.[36] A CT is considered prolonged if it is longer than the normal plasma CT. The correction of the prolonged test result (PT/APTT) by the normal plasma (Table 36-3 ★) indicates a factor deficiency. The normal plasma replenishes the deficient factor in the patient's plasma. If a factor deficiency is detected, specific factor assays should be performed to identify and quantitate the specific factor's activity.

The lack of correction of the prolonged test by normal plasma indicates the presence of a circulating inhibitor or a specific factor inhibitor. A circulating inhibitor inhibits both the coagulation factors in the patient plasma and in the normal plasma with which it is mixed. The detection of a circulating inhibitor (e.g., lupus anticoagulant) should be followed by specific tests to identify and verify the type of inhibitor.

Some patient plasma contains time- and temperature-dependent inhibitors and exhibit correction immediately after mixing with PNP; after incubation, however, the CT result is prolonged (Table 36-3). These inhibitors are called *temperature-dependent inhibitors* and are characteristic of certain FVIII inhibitors. These slow-acting specific inhibitors can be quantified by performing the FVIII inhibitor assay (Bethesda assay). Lupus anticoagulants tend to act immediately but are occasionally time dependent.

☑ CHECKPOINT 36-4

Given the following coagulation test results, what is the appropriate follow-up or reflex test?

PT	Normal
APTT	Prolonged
Mixing studies	No correction

★ **TABLE 36-3** Differentiation of Factor Deficiency from Circulating Inhibitors Using the Mixing Study Procedure

Deficiency or Inhibitor	Immediate PT or APTT after Mixing	Mix, Perform PT or APTT (after 2-hr incubation at 37°C)
Factor deficiency	Correction	Correction
Lupus anticoagulant or heparin	No correction	No correction
FVIII inhibitor	Correction	No correction
FV inhibitor	No correction	No correction

PT = prothrombin time; APTT = activated partial thromboplastin time

Specific Coagulation Factors

Factor assays are performed to confirm a specific factor deficiency and to determine the actual activity of that factor within the plasma. The basis of a factor assay is the ability of the patient's plasma to correct a prolonged PT or APTT of the known factor-deficient plasma (substrate).[37]

Each clotting factor in the extrinsic (FVII) and common (FII, FV, and FX) pathways can be measured by using the one-stage PT assay. The factor assay measures the CT of a mixture of diluted test plasma (patient or control) and specific factor-deficient substrate plasma, which supplies normal levels of all factors except the one being measured. TF and calcium chloride (PT reagent) are added to this mixture, and the CT is determined.

Each intrinsic pathway factor (FVIII, FIX, FXI, and FXII) can be measured by a one-stage, APTT-based method. As in the testing for extrinsic factors, the specific factor assay for intrinsic factors measures the CT of a mixture of diluted test plasma (patient or control) and a specific factor-deficient substrate plasma, which provides all factors except the one being measured.

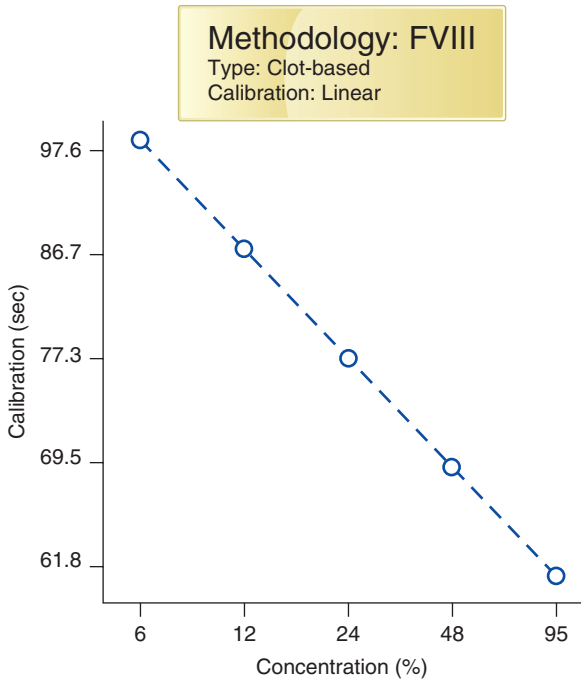
For each clotting factor assay based on the PT or APTT test results, the CTs of factor-deficient substrate plasma containing varying dilutions of a reference pooled plasma (commercially available calibrator that has been assayed against a national or international standard) are used to plot a standard curve (Table 36-4 ★; Figure 36-5 ■). Most laboratories use automated instruments to perform the assays, and the data are plotted using the preprogrammed software included with the instrument. The CTs of the individual dilutions are plotted against the percentage of factor activity of each respective dilution of the assayed standard. The patient's CT using at least three dilutions (usually 1:10, 1:20, and/or 1:40) with the specific factor-deficient plasma is obtained by performing a PT or APTT test on the mixture. These times are converted to percent of activity from the standard curve. The results of the three dilutions should be linear to the standard curve, showing that no "inhibitory effect" is seen. If the results are not linear, an inhibitory effect, which can be seen with lupus anticoagulants, is suspected. The same test is also performed on normal and abnormal controls. The reference interval for most clotting factors is approximately 50–150%. Each laboratory must determine

★ **TABLE 36-4** Dilutions for Factor Assays

Tube	Buffer (mL)	Plasma (mL)	Activity[a] (%)	Dilution[b]
1	0.9	0.1	95.0	1:10
2	1.9	0.1	48.0	1:20
3	0.5	0.5 mL from tube 2	24.0	1:40
4	0.5	0.5 mL from tube 3	12.0	1:80
5	0.5	0.5 mL from tube 4	6.0	1:160

[a]The example is based on a reference plasma value of 95.0%. When a reference plasma with an assayed value other than 100% is used, the activity would be based on its stated value.

[b]Additional dilutions are prepared when patient values are less than 1% activity. Clotting times are determined for each dilution and then plotted on the standard curve. The percent activity is plotted on the x-axis and the clotting time on the y-axis (see Figure 36-5).



Concentration (%)	Measure (sec)	M/D	Interpol (%)
95	61.8		95
48	69.5		48
24	77.3		24
12	86.7		12
6	97.6		6

FIGURE 36-5 Factor activity curve using an FVIII reference plasma activity of 95.0%. If a reference plasma with an assayed value other than this were used, the activity would be based on its stated value. Additional dilutions are prepared when patient values are possibly less than 1% activity. The factor activity curve is prepared by plotting the CT in seconds for each reference plasma dilution on the y-axis and the percent factor activity for each dilution on the x-axis. The CT of at least two patient dilutions is measured, and the percent of activity is interpolated from the factor activity curve. The graph includes the r value and the slope information along with the dilution, CT, and measured values. M/D: M is to record when the operator performs a manual rerun of the calibration point and D is a record for a point deletion if necessary.

CT = clotting times
Modified from the STA-R Evolution coagulation analyzer.

specific factor reference intervals for the reagent/instrument system in use.

The normal levels of some clotting factors (e.g., FIX, FXI, and FXII) are quite low at birth (Table 34-14). Therefore, to distinguish newborn factor deficiencies from normal newborn levels, the laboratory testing must be accurate when assaying samples with levels <10%. In laboratories where hemophilic patients (FVIII or FIX deficiency) are routinely evaluated, the ability to differentiate between factor levels of <1% and 2–5% is essential. This can necessitate the use of lower range calibration curves to evaluate severely decreased levels of coagulation factors.

Commercial chromogenic kits are available to test for FVIII, FX, and other clotting factors. FVIII exists in the plasma as a complex with VWF. Testing involves FVIII activation by thrombin with FVIIIa acting as a cofactor in the conversion of FX to FXa by FIXa in the presence of calcium and phospholipid. The amount of FXa generated is determined by using a specific chromogenic substrate for FXa and is directly proportional to the amount of FVIII in the citrated sample.

The chromogenic FX method is a useful tool in managing patients with lupus anticoagulants who are receiving warfarin therapy and individuals being treated with direct thrombin inhibitors. This method also is used when the conventional PT/INR might not be suitable for individual patients. The method is a two-stage assay. In stage one, the activator Russell viper venom activates FX to FXa in the presence of calcium. In the second stage, the generated FXa hydrolyses the chromogenic substrate S-2765, liberating the chromophoric group pNA. The color is then read photometrically at 405 nm. The generated FXa and thus intensity of color is proportional to the quantity of FX in the sample that is over the assay range.

Currently, these chromogenic kits are less commonly used in clinical laboratories because of their high cost and the ready availability of the one-stage FVIII assay. However, clot-based assays can be affected by specific, nonspecific, and global factors such as heparin, whereas the chromogenic assays are not subject to those interfering substances.[38–40]

The measurement of antithrombin, protein C and protein S, and plasminogen activator inhibitor (PAI)-1 are also available as chromogenic assays and can usually be performed with the same instrumentation.

☑ CHECKPOINT 36-5

What is the function of the factor-deficient substrate reagent and the reference plasma in a factor assay?

Reptilase Time (RT)

The RT is not affected by the presence of heparin in the sample. It is used in conjunction with the thrombin time (TT) to detect heparin contamination of the sample. In the presence of heparin, the RT is normal, whereas the TT is increased. The RT is prolonged in dysfibrinogenemia and in the presence of fibrin degradation products and paraproteins (Table 36-5 ★).

Reptilase is a serine protease from the venom of the *Bothrops atrox* snake. This thrombinlike enzyme cleaves fibrinopeptide A from fibrinogen, whereas thrombin cleaves both fibrinopeptide A and B. The addition of reptilase to PPP initiates clot formation, which can be detected by optical or electromechanical methods using manual, semiautomated, or automated devices. The general reference interval for a reptilase time is 18–22 seconds. An increase to 25 seconds or longer is considered significant and indicates

★ **TABLE 36-5** Differentiation of Conditions Associated with a Prolonged Thrombin Time Using the Reptilase Time

Condition	Thrombin Time	Reptilase Time
Heparin contamination	Prolonged	Normal
Dysfibrinogenemia	Prolonged	More prolonged
Presence of fibrin degradation products	More prolonged	Prolonged

dysfibrinogenemia, hypofibrinogenemia, or afibrinogenemia. Because reptilase is insensitive to heparin, the test can be used to detect hypofibrinogenemia or dysfibrinogenemia when the patient is receiving heparin.

Prekallikrein Screening Test

Individuals with a PK deficiency (also known as *Fletcher factor deficiency*) have a prolonged APTT (Chapter 34). Correction of the prolonged APTT of the patient's citrated plasma to normal or near the upper limit of its normal reference interval after a 10-minute incubation period (before adding calcium chloride) suggests PK deficiency. The longer incubation period increases contact activation of FXII in the absence of PK. The normal control plasma APTT should remain within or near its control value following the extended incubation period. Kaolin, celite, or silica can be the activator of choice for the APTT reagent. Prolonged incubation with ellagic acid might not correct the APTT in PK deficiency.[41] PK deficiency can be confirmed by performing a specific factor assay using PK-deficient substrate. As with the FVIII assay, a quantitative chromogenic substrate assay can quantify the level of PK in plasma samples.[42]

Factor XIII (Screening Test)

FXIII activity is necessary for the formation of a stable fibrin clot that occurs by forming covalent bonds between fibrin monomers. Routine screening tests (PT, APTT) detect clot formation, not cross-linking, and thus are not sensitive to FXIII deficiency. Determination of FXIII should be performed for a patient with clinical symptoms of delayed bleeding or a bruising disorder in whom the screening tests are normal. The screening test for FXIII deficiency is based on the observation that the fibrin clot has increased solubility because of the lack of crosslinking of the fibrin polymer in the absence of FXIII.

The patient's PPP is mixed with 0.025M calcium chloride and allowed to clot for 1 hour at 37°C. The clot is removed and placed in another tube containing 5M urea in a 37°C water bath. Normal control plasma is tested simultaneously. The dissolution of the patient's clot within the 24-hour period indicates an FXIII activity of <1–2% (Chapter 34). The normal control plasma clot is insoluble in these agents. Patients with levels of 1–2% FXIII produce clots that typically dissolve within the first 30 minutes,[43,44] the level at which clinical manifestations of FXIII deficiency occur.

More accurate quantitative assays for FXIII that are more sensitive than the screening test described here have been developed. These assays have not gained widespread acceptance because they are relatively infrequently requested and are most likely performed in research or reference laboratories.

Laboratory Tests for von Willebrand Factor (VWF)

The diagnosis of VWD involves determining the level of VWF by both functional and antigenic methods (Chapter 34). A large number of analytical variables affect the accuracy of these tests for VWF. The first is the variability of laboratory findings in patients with VWD. Serial studies of VWD patients show variability in VWF results over a 24-month span. Because some patients with VWD occasionally have VWF antigen and/or activity (VWF:Ag; VWF:A) levels within the normal reference interval, a single evaluation cannot rule out VWD diagnosis.[45,46]

The second variable is that the results of screening tests (bleeding time, PFA-100®, and APTT) can be normal in patients with type 1 VWD. When persons are studied using both the BT and PFA-00®, the results are not always concordant.[47–49] Typically, these patients have FVIII:C, VWF:A, and VWF:Ag levels of approximately 45–55%, usually producing normal screening test results. Therefore, patients with significant bleeding histories should be further tested with specific assays (VWF:A and VWF:Ag) even when screening test results are within normal limits. VWD cannot be ruled out on the basis of a normal APTT and platelet function screening tests.[50]

The third variable is the fact that an endogenous release of adrenaline can result in a transient increase in plasma FVIII and VWF, so a patient who has VWD type 1 can appear normal. The stress of a difficult phlebotomy, especially in anxious young children, can double or triple the VWF level, making it impossible to rule out VWD without repeated testing.[50] The patient should be as calm as possible before the blood is collected for von Willebrand testing.

The fourth is a technical variability involving the processing of the citrated sample. Samples that are not centrifuged properly at 1500 g for 5–10 minutes can have residual platelets in the plasma as high as 30,000 to 40,000/mcL. Freezing this plasma releases platelet proteases, altering VWF multimeric structure and resulting in an apparent increase in the VWF antigen level and decrease in the VWF activity.[50] Only citrated PPP samples with platelet counts <10,000/mcL should be used for von Willebrand testing.

The fifth variable is the reference standard used in the clinical laboratory, which can affect the various assays for VWF. Clinicians often use the ratio of VWF:Ag to VWF:A to subclassify type 2A and 2M variants. Therefore, it is imperative that both of these assays be interpreted against an acceptable reference plasma sample.

The sixth variable is the influence of the patient's blood type on VWF plasma level. Individuals with blood group AB have a 60–70% higher level of VWF than those with blood group O. As a result, some laboratories interpret VWF levels using specific reference intervals for blood types[51] (Table 36-6 ★).

A variety of clinical disorders, including pregnancy, inflammatory disease, and use of oral contraceptives, also can cause increased levels of VWF.[52] Transient elevations in VWF levels could mask actual low or borderline levels.

The treatment of choice for the classic type 1 VWD is desmopressin (desamino-D-arginine vasopression [DDAVP]), which causes a two- to fivefold increase in VWF plasma level in most patients with type 1 disease.[53,54] A test dose of DDAVP is given to a patient prior

★ **TABLE 36-6** Relationship between Blood Groups and von Willebrand Factor Level

Blood Groups	von Willebrand factor level (%)
O	74.8
A	105.9
B	116.9
AB	123.9

to scheduled surgery to measure his or her response. Citrated plasma samples are drawn preinfusion as well as 30 minutes and possibly 4 hours postinfusion and are assayed for FVIII activity, VWF:Ag, and VWF:A. Care must be taken in handling, centrifuging, and labeling these timed samples.

von Willebrand Factor Activity (VWF:A)

Ristocetin was used as an antibiotic until the early 1970s when it was recognized to cause thrombocytopenia in normal individuals but not in certain individuals with bleeding problems. Although no longer used clinically as an antibiotic, laboratories use it as a diagnostic tool in testing for VWD. The test also is referred to as the **ristocetin cofactor (RCoF) assay** (see the "Platelet Aggregometry" section).

In the presence of ristocetin, VWF induces platelet agglutination that is measured using a platelet aggregometer (RIPA). Decreased RIPA in the PRP occurs in patients with VWD caused by VWF deficiency or BSS (VWF receptor/GPIb/IX deficiency; Chapter 33). The RIPA assay can also be used to differentiate type 2A from type 2B VWD. In type 2B VWD, the qualitative abnormality of VWF results in enhanced binding to the GPIb/IX receptor on platelets, resulting in platelet agglutination at reduced concentrations of ristocetin.

von Willebrand factor activity (VWF:A) can be quantitated using ristocetin to induce VWF agglutination of normal formalin-fixed platelets suspended in patient plasma (the source of VWF). This modification of the RIPA referred to as the *RCoF assay* eliminates the variability of the patient's GPIb/IX receptor on his or her platelets. The slope of the agglutination is plotted versus the percent VWF activity of a reference plasma. Other modifications of the assay use the time elapsed expressed in millimeters on the graph instead of slope of agglutination.[55] A reference (standard) curve is prepared by making dilutions of reference plasma (100%, 50%, 25%, and 12.5% VWF level) and performing the assay on each dilution. The rate of agglutination or the slope of the curve is plotted against each concentration. The control and each patient sample are tested at two separate dilutions, and the resulting slopes or rates are calculated from the reference curve; the results are averaged and reported as a percentage of activity of VWF:A. This assay is very time consuming and labor intensive and has a high coefficient of variation (CV) in testing.[56–59]

A new assay that uses a latex particle-enhanced immunoturbidometric assay (LIA) to quantify VWF:A is available for testing on automated laboratory instruments. The test reduces the variability resulting from the laboratory professional's technical skill. The VWF:A is determined by measuring the increase of turbidity produced by the agglutination of the latex reagent. A specific anti-VWF monoclonal antibody directed against the GPIb binding site on VWF is adsorbed to the latex reagent and reacts with the VWF in patient plasma. The degree of agglutination is directly proportional to the activity of VWF:A in the sample and is determined by measuring the decrease of transmitted light caused by the aggregates.[59]

The general reference interval for VWF:A is 60–150%. VWF:A is usually decreased in plasma from patients with all three types of VWD and the platelet-type pseudo-VWD (Chapter 34, Figure 34-2); type 3 has the lowest level. Unlike the RIPA test that uses patient PRP, which is abnormal in both BSS and VWD, the VWF:A (VWF:RCoF; VWF:A, LIA) assay is normal in patients with BSS.

Normal fluctuations occur with the four diagnostic tests just discussed. Large variations occur in test results in normal individuals as well, emphasizing the need for commercial reference plasmas to be standardized against international standards when preparing the standard curve for all VWF testing.

The laboratory professional should understand the test results regarding hemophilia versus VWD (Table 34-5) and be able to evaluate each patient's test results, repeat them, and perform verifications as needed.

von Willebrand Factor Antigen Assay (VWF:Ag)

As with clinical testing for most bleeding disorders, the antigenic measurement for VWF protein was available before functional assays. Zimmerman first used the "rocket" immunoelectrophoresis technique of Laurell[60,61] to measure VWF:Ag. The assay is time consuming, and measuring and calculating decreased levels is difficult.

Another method for VWF:Ag is an ELISA assay called the *sandwich technique*.[62] Advantages of the ELISA test are increased reproducibility, accuracy at very low levels, and reduced time needed to obtain results compared with the Laurell test. The ELISA procedure uses microtiter plates coated with a specific antibody to VWF produced in rabbits and a second antibody reagent that is enzyme-labeled with alkaline phosphatase or peroxidase. A substrate for the enzyme used is added to produce a color reaction that is read using a microplate reader. This procedure also is in limited use today. An automated, immunoturbidimetric assay for VWF:Ag recently has been developed. The LIA test procedure coats microlatex particles with rabbit antihuman VWF antibodies, producing agglutination when mixed with plasma containing VWF. The extent of agglutination (turbidity) correlates to the level of VWF:Ag present in the citrated plasma sample. The methodology is reliable, easy to automate, and produces results in a timely manner. In addition, it can detect low levels of antigen, thus improving the ability to diagnose variants of VWD. (See this chapter's Companion Resources: techniques used for specialized testing.)

von Willebrand Factor (VWF) Multimer Analysis

Proper treatment of VWD depends on determining the correct disease subtype (Chapter 34). The availability of multimeric analysis has improved diagnosis and treatment of VWD patients. VWF in normal plasma exists as multimers ranging in size from dimers of approximately 600,000 daltons (Da) to very large multimers of up to 20 million Da. Plasma electrophoresis using low concentrations of agarose (0.65%) and staining with radiolabeled antibody to VWF allows the full range of multimers to be visualized (by autoradiography or liminography). DDAVP is the treatment of choice for type 1 VWD but is less effective in type 2 and not effective at all in type 3.

Therefore, establishing the correct VWD subtype by multimeric analysis is important in determining the appropriate therapeutic approach.

Multimeric analysis is also useful in diagnosing thrombotic thrombocytopenic purpura (TTP) in which unusually large multimers of VWF (UL-VWF) are observed. Normally, ultralarge multimers are cleaved by the VWF protease, **a** **d**isintegrin-like **a**nd **m**etalloprotease with **t**hrombo**s**pondin (ADAMTS-13) (Chapter 35). The presence of UL-VWF indicates a reduced or absent ADAMTS-13 activity.

Because these multimeric analyses are difficult to perform and interpret and require a very pure and specific antibody, specialized reference laboratories or coagulation centers usually perform VWF multimeric analyses.

Collagen-Binding Assays for von Willebrand Disease (VWD)

Collagen-binding assays (VWF:CB) are specialized assays used to differentiate VWD type 2A and 2B from type 2M. The ELISA assays for collagen binding were initially considered to be potential replacements for the VWF:RCoF assay because both tests are sensitive to a reduction in high-molecular-weight multimers of VWF. However the VWF:CB assays do not reflect the interaction between VWF and GPIb; thus, they cannot universally substitute for VWF:RCoF assays. Performing both VWF:CB and RCoF assays increases the ability to differentiate VWD type 2 variants (discrimination of VWD types 2A or 2B from type 2M).[52] Whereas all three subtypes (2A, 2B, 2M) give abnormal RCoF results, only types 2A and 2B give abnormal VWF:CB results; type 2M patients have normal VWF:CB.

A Disintegrin-Like and Metalloprotease with Thrombospondin (ADAMTS-13)

ADAMTS-13, a VWF-cleaving metalloprotease, controls platelet aggregation by cleaving ultralarge high-molecular-weight VWF multimers. A deficiency or mutation of ADAMTS-13 results in the persistent attachment of large VWF multimers to endothelial cells and adherence of platelets. This leads to the formation of platelet aggregates/thrombi and is the cause of TTP. ELISA, fluorescence resonance energy transfer (FRET), and various other direct or indirect assays are available for measuring ADAMTS-13 activity, antigen, or autoantibody levels. Citrated, platelet-poor plasma samples are used for testing. To date, only a few laboratories have the experience to perform assays for this rare bleeding disorder. These assays are generally performed in facilities that have a great deal of expertise and patient populations to support this testing.[63]

Identification of Inhibitors

The most common circulating inhibitors seen in the hemostasis laboratory are lupus anticoagulants (LAs) or antiphospholipid antibodies (aPLs) and FVIII inhibitors. Various procedures are used to detectLA/aPLs because no single definitive assay is available.[64] Because LAs/aPLs are transient, detection is often difficult. The measurement of the inhibitor to FVIII is determined with the FVIII inhibitor assay (Bethesda method).

Lupus Anticoagulants/Antiphospholipid Antibodies

The inhibitors originally described as *lupus anticoagulants* are now recognized to be part of a more diverse group of inhibitors collectively referred to as aPLs (Chapter 35). They are immunoglobulins, usually of the IgG class, that are directed against the protein component of protein-phospholipid complexes. In vivo, aPLs are associated with a thrombotic tendency; in vitro, they prolong phospholipid-dependent clotting assays. The diagnosis of aPL requires the demonstration of (1) an abnormality of phospholipid-dependent coagulation reactions, (2) an abnormality that results from the presence of an inhibitor rather than a factor deficiency, and (3) the phospholipid-dependence of the inhibitor.

The quality of the patient sample (PPP) greatly affects the integrity of the testing. Procoagulant phospholipids (originating from platelets) can sometimes be in the patient plasma or the normal plasma used for the mixing studies. If present, they can neutralize weak lupus anticoagulant activities and produce false negative results. Thus, plasma samples should be centrifuged for 15 minutes (2000 g) after which the top two-thirds of the plasma are carefully removed (so as not to disturb the cells) using a plastic transfer pipette. The plasma is then transferred to a second plastic tube and recentrifuged for an additional 10 minutes (>2500 g). If testing is to be delayed, the PPP should be transferred to a cryovial and stored at −70°C. Before testing, the frozen plasma should be thawed in a 37°C water bath for 5 minutes to avoid the formation of cryoprecipitate.

No single test is sensitive for all LA/aPLs. Thus, two different screening tests based on different assay principles should be performed. If more than two types of screening tests are used, the risk of a false positive result increases to unacceptable levels.[64] The **dilute Russell viper venom time (dRVVT)** test is recommended as the initial screening test. The procedure is considered extremely robust and has been shown to be specific for detecting the presence of an LA/aPL in subjects at an elevated risk of thrombosis.

The APTT has historically been used to screen for LA/aPLs, although APTT's sensitivity to LA/aPL depends on the reagent.[65] Any APTT assay using silica as an activator and a low phospholipid content is the second choice of screening methods. APTT reagents with ellagic acid activators are not recommended for detection of LA because they lack sensitivity for the presence of LA. Many modifications to the APTT, including the use of lower concentrations of phospholipids in the test reagent, have been recommended in an attempt to increase its sensitivity. Laboratory professionals must evaluate their APTT reagents as to the importance of LA/aPL sensitivity versus the importance of sensitivity to specific factor deficiencies. In cases for which the screening APTT is not prolonged but the patient presents with a positive history of thrombophilia, the physician should proceed with designated testing for LA/aPL. The presence of an LA/aPL should be considered if one of the two screening tests gives a positive result.

According to the guidelines developed by the International Society on Thrombosis (ISTH) 2009,[64] laboratory evaluation for the diagnosis of an LA should include the following:

- Double centrifugation to ensure PPP, $<10.0 \times 10^3$/L; quick-freeze if testing is delayed; thaw at 37°C.
- Two clot-based assays employing separate clotting principles as screening tests. The dRVTT assay is the most robust. The other assay can be low-phospholipid APTT with silica activator. APTT with kaolin activator is problematic in automated coagulometers; ellagic acid is insensitive to LAs; both are contraindicated.

- Performance of a TT to detect therapeutic heparin and absorb heparin or collect sample at another time in the absence of anticoagulant therapy.
- Preparation of PNP for mixing studies is prepared "ad hoc" (homemade) by double centrifugation to ensure it is PPP. PNP must provide 100% activity for all clotting factors. Commercial lyophilized or frozen normal plasmas can be used if they fulfill these specifications. A 1:1 mixture of patient plasma with PNP should be tested without preincubation.
- Development of cutoff values using the local reagent/coagulometer combination on at least 40 adult healthy donors <50 years old.
- Performance of mixing studies on plasmas from healthy donors mixed with the PNP at a 1:1 proportion. The cutoff is the 99th percentile of the distribution of results from the healthy donors or an index of circulating anticoagulant (ICA) computed as follows:

$$\text{ICA\%} = [(\text{Clot time of patient-PNP mixture} - \text{Clot time of PNP})/\text{Clot time of patient}] \times 100$$

- Performance of the confirmatory test by increasing the concentration of PL in the screening test. Either bilayer- or hexagonal-phase phospholipid can be used. Frozen/thawed platelets are not recommended because of poor batch-to-batch consistency.
- Confirmatory testing that requires testing healthy donor plasma at both low PL concentrations (screen) and high PL concentrations (confirm) to obtain CTs. The confirmatory test cutoff is the value corresponding to the mean of the individual percentage corrections defined as follows:

$$[(\text{Screen CT} - \text{Confirm CT})/\text{Screen CT}] \times 100 = \text{\% correction}$$

- The result is confirmation of LA if the percentage correction is above the cutoff value defined previously.

The following tests are not recommended for LA screening: dilute PT, Ecarin and Textarin times, and kaolin CT.

Dilute Russell Viper Venom Time (dRVVT) Test

The dRVVT test uses a commercial preparation of the venom from the Russell viper (*Daboia*) to activate FX. The dRVVT test is based on the premise that LA/aPL activity is enhanced in the presence of reduced phospholipids. The reagent contains dilute Russell viper venom (dRVV), calcium chloride, and phospholipids. The reagent is added to patient PPP, and Russell viper venom (RVV) activates FX, resulting in clot formation. If LAs/aPLs are present, the patient's dRVVT is longer than that of the normal control. The ratio of the patient's CT to the CT of the normal control is determined. The normal ratio is usually less than 1.2. A confirmatory test also uses RVV but with a higher phospholipid concentration. The final result is reported as a ratio of the two clotting tests (high and low phospholipid concentration), which is compared with the values of a reference population.[66]

The dRVVT appears to be more sensitive to aPL antibodies that react with β2-glycoprotein I (Chapter 35).

Hexagonal Phospholipids

The hexagonal-phase phospholipids (HPPs) test uses egg phosphatidylethanolamine in a hexagonal-phase configuration. The HPP assay is based on the fact that many aPL antibodies specifically recognize the HPP configuration as an antigenic epitope. Addition of HPPs to the reaction mixture neutralizes the inhibitory effect of the aPL antibodies but does not neutralize most factor-specific antibodies.[67] The test (Staclot LA, Diagnostica Stago) is performed by incubating the test plasma at 37°C with and without the HPP reagent. An APTT is performed on both of these incubations using an aPL-sensitive reagent. If aPL antibodies are present in the test plasma, the HPPs would neutralize them, resulting in a shortened CT for the tube containing it compared with the tube without HPPs. By comparing the two CTs, the presence of aPL antibodies can be identified. This assay has two advantages: (1) The LA-sensitive reagent contains a heparin inhibitor that makes the test system insensitive to heparin levels up to 1 IU/mL and (2) the Staclot LA procedure adds normal plasma to the test system to correct any prolongation of CT from factor deficiencies that might be present. The correction of the APTT result varies, depending on the instrument used. Photo-optical systems have shorter times than electromechanical devices.[68] Each laboratory should establish its own cutoff by determining the range of $\pm$ 4SD from the mean of a normal population. Diagnostica Stago lists a cutoff of 8 seconds for electromechanical systems. The correction is consistent with the presence of a phospholipid-dependent inhibitor.

Specific Factor Inhibitor Assay (Bethesda Titer Assay)

The Bethesda titer assay is primarily used to quantify inhibitory antibodies against infused FVIII, but it can be adapted to assay any specific inhibitor of coagulation factor proteins. Detection of an inhibitor plays an important role in treating subjects with hemophilia A. Inhibitory antibodies to FVIII cause major complications for hemophilia A patients and can be found as acquired inhibitors in patients without hemophilia.

In spite of improvements in component therapy products for hemophilia A, the development of inhibitors to FVIII continues to be a major problem in treating this disorder.[69] Inhibitors to FVIII develop in 3–52% of patients with severe hemophilia A. Polyclonal alloantibodies neutralize the FVIII transfused from various clotting factor components, possibly leading to severe bleeding and problems in managing surgical procedures.

Specific inhibitors occur in 10–15% of hemophiliacs at any time after the first infusion of factor concentrate. To detect the presence of an inhibitor in a hemophiliac, the patient's response to treatment (FVIII concentrates, both human and recombinant) is monitored by FVIII:C assays. FVIII inhibitors can be identified in special coagulation facilities by using clot-based methods or ELISA assays.

The Bethesda inhibitor assay is the most commonly used assay to quantify antibodies to FVIII. This assay quantifies FVIII activity that

is left after the patient's plasma is mixed with an equal volume of PNP containing a known FVIII activity and incubated for 2 hours at 37°C. The incubation allows the inhibitor in the patient's plasma to neutralize the FVIII in the PNP. Increasing the dilutions of patient plasma also can be prepared using imidazole buffer to dilute the inhibitor and then mixed with PNP. A control consisting of PNP mixed with imidazole buffer (1:1 mix) is set up and incubated to check for the deterioration or thermolability of FVIII. An FVIII assay is performed on all incubation mixtures, including the control.

The presence of an FVIII inhibitor inactivates the FVIII in the PNP, and any residual FVIII can be measured. The patient mixtures are corrected for the amount of FVIII loss seen in the control tube (normal plasma + buffer). The residual FVIII values (FVIII level measured in each of the incubated patient dilutions plus control) between 25–75% activity are then converted to Bethesda units (BUs) using the standard Bethesda chart (Web Table 36-5). Results for each of the dilutions (BUs) are multiplied by the reciprocal of the dilution and averaged. In the incubation mixture, 1 BU = 50% residual FVIII.[70]

In patients with high inhibitor levels, multiple dilutions of patient plasma are needed to produce residual FVIII activity that can be read from the standard curve. The results are then averaged. When the results show that the inhibitor level appears to rise as the plasma is more diluted (Web Table 36-6, patient C), the patient appears to have complex reaction kinetics, and the inhibitor level is calculated from the lowest dilution that gave a corrected percent residual FVIII closest to 50%.

Nijmegen modified the original Bethesda assay by buffering the PNP to a pH of 7.4. He replaced the imidazole buffer in the control mixture with immunodepleted FVIII-deficient plasma (1:1 mix) and in each dilution of patient plasma. These modifications help to maintain the pH of the mixtures for the 2-hour incubation and stabilize the FVIII present in the PNP.[71] They also improved the specificity and reliability of inhibitor detection in patients with low-titer FVIII inhibitors.

Testing for a specific inhibitor is always performed on a preinfusion plasma sample (a trough sample that should have an FVIII:C activity of <1.0%, typically >72 hours after the last FVIII infusion). If the patient has an inhibitor, the FVIII that is subsequently infused rapidly clears, resulting in an FVIII level of <1% usually within 24 hours.[72] Presence of residual infused FVIII can interfere with an inhibitor assay and produce a false negative result. Additionally, in patients with high levels of inhibitors, there can be an effect when assaying other clotting factors. For example, a high FVIII inhibitor level in a patient who is being tested for FIX deficiency could cause the FVIII level in an FIX-deficient reagent plasma to decrease, causing an abnormal factor assay result. Thus, it would appear that the patient had an FIX deficiency in addition to FVIII even if the FIX in the patient was within the reference range.

☑ CHECKPOINT 36-6

Why are excess lipids able to neutralize lupuslike anticoagulants?

LABORATORY INVESTIGATION OF THE FIBRINOLYTIC SYSTEM

Fibrinolysis is the last stage of the coagulation mechanism and involves the breakdown of fibrin clots into degradation products. This process begins after fibrin polymerization and cross-linking have taken place. In normal subjects, plasminogen, plasmin, TPA, and PAI-1 are incorporated into fibrin clots by binding to lysine amino acids in fibrin via lysing binding sites (LBSs) located in the "kringle domains" of these proteins. A disruption of the equilibrium of the fibrinolytic/coagulation balance by hereditary or acquired disorders can result in fibrinolytic disorders[73] (Web Table 36-7).

Critically ill patients and individuals with certain chronic illnesses can have abnormal fibrinolysis and experience excessive bleeding or thrombosis. Patients treated with fibrinolytic therapy or end-stage liver disease can experience occurrences of elevated fibrinolysis, which can be especially evident at sites of injury or surgery.[74] Disorders associated with defective fibrinolysis include type 2 diabetes, insulin resistance, sepsis, stroke, and metabolic syndrome (increased blood pressure, high blood sugar, excess body fat around the waist, abnormal cholesterol levels). These conditions can increase the risk for developing coronary artery disease and stroke, deep vein thrombosis, and pulmonary embolism.[75,76]

Laboratory assays for analysis of the fibrinolysis/coagulation system are essential for rapid patient diagnosis and treatment. The following is a discussion of a variety of methods that measure components of the fibrinolytic system.

D-Dimer

The D-dimer is a specific fragment resulting from plasmin degradation (lysis) of the fibrin clot, which has been cross-linked by FXIIIa (Chapter 32). It is an excellent marker for DIC with secondary fibrinolysis. However, D-dimers are also elevated in pulmonary embolism, deep vein thrombosis, arterial thromboembolism, recent trauma or surgery, cirrhotic liver disease, renal failure, and hospitalized subjects[77] (Chapter 33).

The D-dimer test is an immunoturbidometric method that utilizes monoclonal antibodies against the D-dimer fragment, which can be used in three separate methodologies. The first is a semiquantitative assay that uses macroscopic latex agglutination and is usually performed on citrated plasma. The patient specimen is mixed with latex particles coated with monoclonal antibodies (directed against D-dimer) on a glass slide for a specific amount of time. The reaction mixture is observed macroscopically for the presence of agglutination. Most procedures test both undiluted plasma and a plasma sample diluted 1:2 in buffer that allows a semiquantitation of results. Lack of macroscopic agglutination in either sample corresponds to a D-dimer level of <0.5 mcg/mL. Macroscopic agglutination in the undiluted sample but not in the 1:2 dilution corresponds to a D-dimer of 0.5–1.0 mcg/mL. If both samples show agglutination, the result is >1.0 mcg/mL. Normal individuals have <0.5 mcg/mL.

The second methodology is a more sensitive, quantitative technique using the ELISA methodology. Although very sensitive and specific for D-dimer, this technique is time consuming (clinically, results are usually needed quickly) and requires the use of specialized equipment.

The third methodology is also based on the concept of microscopic latex agglutination but is an automated procedure and eliminates human error associated with visual determination of results. Antibody-coated latex particles aggregate in the presence of D-dimer, resulting in the mixture's increased turbidity. The increase in scattered light is proportional to the amount of D-dimer in the sample. This assay is extremely sensitive and has an increased detection range. It is considered to have good negative predictive value; a negative test can be used to rule out deep vein thrombosis (DVT), venous thromboembolism (VTE), and pulmonary embolism (PE).[78,79] The assay, if effectively used, can reduce the use of time-consuming and expensive diagnostic tools such as venography, compression ultrasound, and spiral computed tomography scans for some patients. If the level of D-dimer in the plasma is *not* elevated, no thrombotic process is ongoing, and VTE is not present (e.g., there is a "negative predictive value"). However, if the D-dimer level is elevated, coagulation is occurring, which could happen because of VTE as well as other clinical conditions (Table 36-7 ★). The major difficulty with using the D-dimer assay to exclude VTE is establishing the cutoff value to be used. Each laboratory must establish its own cutoff value rather than relying on manufacturer-provided guidelines (Web Table 36-8). These values need to be developed with input from the clinical staff and re-evaluated periodically. The D-dimer assay should not be used in patients on anticoagulant therapy (heparin or warfarin). Studies have demonstrated that anticoagulants decrease circulating D-dimers and could produce a value below the cutoff, masking the presence of VTE.

Most hospitals that use the D-dimer assay to confirm DIC also test for VTE with it. Because the range of detection necessary for DIC and VTE varies significantly and the two diagnoses utilize different cutoff values, some institutions provide them as two separate tests.

Fibrin Degradation Products

The detection of increased levels of fibrin and/or FDPs in patient blood samples indicates increased fibrinolytic activity (Chapter 32). The patient's blood sample is collected in a special collection tube containing thrombin (to clot the sample and ensure that all fibrinogen has been removed) and a fibrinolytic inhibitor (to prevent in vitro fibrino[geno]lysis). The patient's serum is mixed with latex particles coated with specific antibodies that recognize FDP on a glass slide for a specific amount of time. The reaction mixture is observed macroscopically for the presence of agglutination. The test is semiquantitative with various dilutions made of the patient's serum in buffer. The normal range varies according to the particular test's manufacturer and sensitivity. Most procedures require the preparation of two dilutions for each sample (e.g., 1:2 and 1:8), which allows a semiquantitative determination of FDP. The D-dimer assay has largely replaced FDP assays, and many institutions no longer perform both.

★ **TABLE 36-7** Partial List of Non-Venous Thromboembolism Causes of Elevated D-Dimer

DIC	Pregnancy
Trauma	Cancer
Surgery	Diabetes
Hematoma	Thrombolytic therapy
Arterial thrombosis	Older age
General hospitalization	

Euglobulin Clot Lysis

A very elementary procedure, the euglobulin clot lysis (ECL) was used before specific antibodies to fibrinolytic products were available. The euglobulin protein fraction (containing fibrinogen, plasminogen, and plasminogen activators [tPA], but no fibrinolytic inhibitor [PAI-1]) is precipitated from plasma in a weak acid solution. The precipitate is redissolved, clotted with thrombin, and observed for clot lysis at 37°C. The fibrin clot generated by thrombin serves as the substrate for plasmin generated from plasminogen by plasminogen activators (also activated by thrombin). The end point is the time in minutes required for complete degradation of the clot into small fibrin strands or particles.

The ECL is normally >90 minutes. Shortened lysis times indicate hyperfibrinolysis. Shortened lysis times can occur in fibrinolysis secondary to intravascular coagulation (DIC), in liver disease because of poor clearance of activator, and in thrombolytic therapy because of the therapeutic administration of a plasminogen activator. The ECL assay is not reliable for assessing thromboembolic states.

LABORATORY INVESTIGATION OF HYPERCOAGULABLE STATES

Individuals who have repeated thrombotic episodes most likely are in a **hypercoagulable state**. Laboratory tests can assist in determining whether the thrombosis is hereditary or an acquired abnormality of the hemostatic system.

An improved understanding of hemostasis and the regulatory roles of naturally occurring anticoagulants has produced major advances in identifying some of the hereditary defects associated with hypercoagulability. Testing can be performed either by clottable, chromogenic, ELISA, or latex immunoassay functional assays. These techniques are described in this chapter's Companion Resources.

An important issue is when to test a patient who has had a thrombotic event. For a venous thrombosis but not an arterial (platelet-derived) thrombosis, the standard accepted approach is to treat the patient appropriately and test later. The diagnostic workup is delayed until the patient is stable, no longer hospitalized with a thrombotic event (which consumes coagulation factors and inhibitors, Chapter 35), and not receiving anticoagulant therapy. Warfarin (vitamin K antagonist), heparin, direct thrombin inhibitors (Agatroban, Dabigatran), or FXa inhibitor (rivaroxaban) all can interfere with clottable assays that can be used to monitor these anticoagulation therapies. When a subject is receiving replacement therapy such as fresh frozen plasma (FFP) or other blood components, they also should not be tested for hypercoaguability. Testing for protein C and protein S (vitamin K-dependent proteins) while a patient is taking warfarin or during an active thrombotic process can lead to falsely low activity and/or antigen values and therefore can result in the misdiagnosis of an inherited hypercoagulable state. Repeat studies are often needed, and family studies are usually required to confirm a diagnosis.

Antithrombin (AT)

AT, previously called *antithrombin III*, has powerful and immediate antiprotease action in the presence of heparin. This interaction is the basis for the poor clinical response to heparin if the patient has significantly decreased levels of AT. A variety of assays can measure AT.

The chromogenic assay is a functional two-part assay measuring AT activity in the presence of heparin (Web Figure 36-3). In the first part, plasma is incubated with a known excess of thrombin in the presence of heparin (heparinized buffer). In its presence, AT neutralizes a proportional amount of the thrombin. The second part of the assay determines the residual thrombin activity by its enzymatic activity on an appropriate substrate (fibrinogen tagged with a chromophore, such as p-Nitroanilin [pNA], which produces a yellow color when released that is measured at 405 nm). The amount of thrombin neutralized in the first reaction step is proportional to the amount of AT present in the test sample; thus, the residual amount of thrombin (as measured by the pNA release) is inversely proportional to the test sample's AT level. A reference (standard) curve is prepared by plotting the AT activity (%) for each reference plasma dilution prepared against its corresponding absorbance. The results for patients and controls are read from this curve using respective absorbance readings. The general reference interval for AT for either clottable or chromogenic assays is 78–126%.

The other common functional assay is a clot-based method in which the clotting of fibrinogen rather than the cleavage of a synthetic chromogenic substrate is the assay endpoint. Plasma is defibrinogenated (fibrinogen removed) and incubated with heparin and thrombin. Residual thrombin is determined by transfer to a standardized fibrinogen solution, and the time for clotting to occur is measured. As in the chromogenic assay, the CT is directly proportional to the concentration of AT in the patient's plasma: the higher the AT level, the higher is the amount of thrombin neutralized in the first step, resulting in a lower level of residual thrombin and a prolonged CT in the second step.

Immunological methods measure the concentration of the AT protein using EIA, ELISA, radial immunodiffusion (RID), or microlatex particle immunological assays (LIA tests, automated procedures available).

Inherited deficiencies of AT can involve a decrease in both AT protein and activity level or the presence of a dysfunctional protein (Chapter 35). In addition to congenital deficiencies (which are very rare), acquired deficiencies occur in DIC, liver disease resulting from decreased synthesis, and nephrotic syndrome. A number of clinical disorders can be associated with reductions in the plasma concentration of AT, sometimes making it difficult to establish a definitive diagnosis of an inherited deficiency. Low levels on an initial screen should be confirmed at a later date. All hereditary deficiencies identified to date have occurred in heterozygous individuals.

Anticoagulation of patients also affects testing for AT. The administration of heparin decreases plasma AT levels presumably by accelerated clearance of the heparin-antithrombin complex. Thus, the evaluation of plasma samples during heparinization can lead to an erroneous diagnosis of AT deficiency. Plasma AT concentrations are occasionally elevated into the normal range by warfarin in individuals with AT deficiency. Confirmation of the hereditary nature of an AT deficiency can require investigating other family members.

Protein C/Activated Protein C (APC)

Functional assays for protein C (PC) include measuring amidolytic activity using a chromogenic substrate or PC anticoagulant activity in a clot-based assay. Chromogenic assays can be less informative than clot-based assays when screening for PC defects. Several individuals have been described with normal PC antigen (immunologic) measurements and reduced PC anticoagulant activity in clot-based assays but normal amidolytic activity in chromogenic assays.[80] These individuals could have defects in the ability of APC to interact with platelet membranes or the FVa or FVIIIa substrates and thus would not be detected by the amidolytic assay.[80]

APC is an anticoagulant that inactivates FVa and FVIIIa. In the PC assay, a reagent containing snake venom (*Agkistrodon c. contotrix*), protac (a particulate activator for the activation of protein C), and platelet factor 3 activate both PC and the contact factors of the intrinsic pathway. The patient's plasma is incubated with PC-deficient plasma (to compensate for any factor deficiency other than PC) and an APTT reagent containing the activator; calcium chloride is added and the time for clot formation is measured. Activation of PC, resulting in the inactivation of FVa and FVIIIa in the control and patient samples, causes a prolongation of the modified APTT. The longer the modified APTT, the more functional PC is present in the plasma sample. A reference (standard) curve is prepared by plotting the PC activity for each reference plasma dilution assayed in conjunction with the patient samples against its CT. The results for patients and controls are obtained from this curve using the respective CTs. The general reference interval for PC is 60–150%.

In spite of the limitations noted, the chromogenic assay for PC is widely used. This assay incubates PC with a specific activator; the amount of APC measured is based on APC's enzymatic activity on a chromogenic substrate. The enzymatic activity releases pNA from the chromogenic substrate, and pNA is measured spectrophotometrically at 405 nm. The absorbance of pNA is directly proportional to the amount of APC. A reference (standard) curve is prepared by plotting the protein C activity (percentage) for each reference plasma dilution against its corresponding absorbance. The results for patients and controls are obtained from this curve using the respective readings.

The diagnosis of PC deficiency is complicated for patients on oral anticoagulation therapy. Warfarin therapy reduces functional and, to a lesser extent, immunologic measurements of PC. Several researchers have proposed using the ratios of PC antigen to FII or FX antigen. However, this does not identify patients with type II PC deficiency (low activity, normal antigen). This approach of using ratios can be used only for patients in a stable phase of oral anticoagulation. Other groups have used PC activity assays in conjunction with functional measurements of FVII (a vitamin K-dependent zymogen with a similar plasma half-life). In practice, individuals suspected of having PC deficiency should be investigated after oral anticoagulation has been discontinued for at least 1 week. Family studies are desirable. If discontinuing warfarin therapy is not possible, individuals can be studied while they receive heparin therapy, which does not alter plasma PC levels.[80]

Acquired PC deficiency is found in numerous disease states (Chapter 35). Most individuals with uremia have low levels of PC anticoagulant activity but normal levels of PC amidolytic activity and antigen. This low PC anticoagulant activity has been attributed to a

dialyzable moiety in uremic plasma that interferes with most clotting assays for PC activity.[80]

Protein S

Circulating protein S (PS) exists in two forms: free (40%) and bound to C4b binding protein (60%). Only the free PS serves as a cofactor for APC, enhancing its anticoagulant activity. The functional free form is measured in clot-based assays. Laboratory evaluation of PS also can include assays of total PS antigen (ELISA) and free PS (immunoassays using monoclonal antibody specific for the free form).[81]

Functional PS assay methods are based on the ability of PS to serve as a cofactor for the anticoagulant effect of APC. A typical clot-based procedure for measuring the cofactor activity of PS requires four reagents: (1) PS-deficient plasma to ensure optimal levels of all coagulation factors except PS, (2) purified activated PC, (3) purified activated FVa to serve as a substrate for APC, and (4) calcium chloride. Patient PPP is mixed with PS-deficient plasma. APC and activated FVa reagents are added to this mixture, which is incubated at 37°C. Following incubation, calcium chloride is added to initiate clot formation. The CT is proportional to the PS activity in the sample (i.e., the higher the level of PS, the longer is the CT).

A reference (standard) curve can be prepared by using dilutions of reference plasma representing 100%, 75%, 50%, and 25% PS activity. This activity for each plasma and control is obtained from this curve. The general reference interval for PS is 60–140%.

Like PC clot-based assays, the presence of FV Leiden, APC resistance, elevated FVIII levels, and a lupus anticoagulant can cause false positive PS test results if clotting (functional) assays are used to diagnose PS deficiency.

Inherited deficiencies (autosomal dominant disorder) can involve a decrease in the protein or a dysfunctional protein (Chapter 35). The clot-based procedure for PS detects both its quantitative and qualitative deficiencies, but immunologic methods do not detect qualitative deficiencies.

Acquired PS deficiencies are found in numerous disease states (Chapter 35). Although total PS antigen measurements are generally increased in individuals with nephrotic syndrome, functional assays are often reduced because of the loss of free PS in the urine and elevations in C4b-binding protein levels.[80]

Activated Protein C Resistance (APCR)

The presence of the factor V Leiden (FVL) mutation results in the resistance of FVa to degradation by APC, commonly referred to as *activated protein C resistance* (*APCR*), one of the most common risk factors for thrombosis. The molecular testing for FLV (PCR) is described in Chapter 42.

The clot-based assay is based on the principle that the addition of APC to a plasma sample induces a prolongation of the APTT mediated by the inactivation of FVa and FVIIIa in the plasma sample. The sensitivity and specificity of the screening test has been improved by prediluting the patient plasma with FV-deficient substrate plasma; this permits the evaluation of patients receiving anticoagulants or who have an abnormal APTT from factor deficiencies other than FV. The testing proceeds with the performance of an APTT with and without the addition of APC. The APC ratio is calculated using the CT of the sample with APC (results should be prolonged because of the destruction of FVa and FVIIIa) divided by the CT for the sample without APC. APCR is indicated by a ratio of less than what had been established for the laboratory using a particular instrument and reagent combination, for APCR ratio calculation, see Web Figure 36-4. Acquired conditions such as pregnancy, oral conceptive use, elevated FVIII, and a stroke can also produce APCR.

The APCR functional assay (as described) is a screening test, not a diagnostic test, for FVL. Other rare congenital conditions including FV Cambridge and homozygosity for the HR2 haplotype can result in APCR. Because 10% of individuals with APCR do not have the FVL mutation, clinical diagnosis of FVL requires both the clotting and molecular (PCR) tests for the FVL genetic mutation. If FVL mutation is not identified, PCR-based confirmatory tests for APCR resulting from other genetic mutations are available in research laboratories.

Prothrombin G20210A

Individuals heterozygous for the prothrombin 20210 (FII G20210A) mutation can have prothrombin levels averaging approximately 130%, up to 30% higher than noncarriers (Chapter 42). Individuals with the mutation have an increased risk of thrombosis that seems to be connected to the elevated FII activity. However, because of the overlap with the normal range for FII, clot-based functional assays for prothrombin are unreliable.[82] Therefore, genetic screening of the FII gene is the only reliable way to detect the presence of this phenotype (Chapter 42). Detection methods for the amplified product can utilize specific endonucleases, gel electrophoresis, or fluorescent probing. A multiplex PCR-based assay is available for the simultaneous detection of FVL and FII G20210A.[83]

☑ CHECKPOINT 36-7

In diagnostic laboratory evaluation of a hypercoagulable state such as antithrombin deficiency, why is it important to determine the functional activity of the coagulation protein?

Additional Testing for Thrombosis

Rare abnormalities in the fibrinolytic system can result in thrombosis. The tests in this section can be performed to assist in the diagnosis of these abnormalities. Except for plasminogen, the tests discussed are performed less frequently than those previously described and can require sending samples to a reference laboratory. If only a limited number of these tests are performed at a particular site, they could be prohibitively costly (Chapters 30 and 33).

Plasminogen

Plasminogen levels are measured by using a chromogenic assay based on the conversion of plasminogen to plasmin by an excess of streptokinase (SK), which acts as an activator. The first step in this assay involves incubating patient plasma with a known excess of SK, which forms a complex (plasminogen-SK) that contains

plasminlike activity. The second step determines the amount of the complex by its enzymatic activity on a chromogenic substrate. The enzymatic activity results in the release from the chromogenic substrate of p-nitroanilin (pNA), which is measured spectrophotometrically at 405 nm. The pNA absorbance is directly proportional to the quantity of plasminogen. A reference (standard) curve is prepared by plotting the plasminogen activity (%) for each reference plasma dilution against its corresponding absorbance. The results for patients and controls are obtained from this curve using the respective absorbance readings. The general reference interval for plasminogen levels is 74–124%.

Inherited deficiencies of plasminogen include quantitative ones characterized by decreased antigenic and functional levels as well as qualitative ones characterized by dysfunctional protein (Chapter 35). Acquired deficiencies are associated with DIC, liver disease, and leukemia. Measuring circulating plasminogen levels is useful in monitoring hepatic regeneration of plasminogen after discontinuation of treatment with plasminogen activators and in controlling and adjusting the rate of infusion of FFP being given to the patient.

α_2-Antiplasmin Activity

A chromogenic method to measure α_2-antiplasmin that can be used with microtiter plate, test tube, or automated procedures is available. Incubation of plasma diluted with an excess of plasmin results in a rapid complex formation between the functional plasmin inhibitor (antiplasmin) present in the plasma and the added plasmin. The amount of plasmin activity inhibited is proportional to the amount of plasmin inhibitor in the patient plasma. The residual plasmin hydrolyzes the chromogenic substrate S-2403, liberating the chromophoric group, pNA. The color is observed photometrically at 405 nm. The inhibitor α_2-antiplasmin is important in regulating the fibrinolytic system. Congenital deficiencies are characterized by bleeding occurring some hours after an initial injury. Clotting and wound healing are usually normal, but the hemostatic plug breaks down prematurely. Acquired decreases of α_2-antiplasmin can be observed in liver disease and DIC. Increased levels have been reported during the postoperative period.

Plasma-α_2-Antiplasmin (PAP) Complex

The PAP complex test uses ELISA methodology to detect elevated levels that can occur during thrombotic events, in cases of endogenous hyperfibrinolysis, and during thrombolytic therapy. The blood sample is collected in special precoated plastic tubes containing citrate, aprotinin, and benzamidine. Special specimen processing is required.

Plasminogen Activator Inhibitor (PAI-1)

Both functional and antigenic measurements are available to measure PAI-1, the primary inhibitor of tPA. PAI-1 is assayed using immunoturbidometric, chromogenic, or ELISA methods, which can be performed on automated instruments. This inhibitor displays diurnal variation, so attention must be paid to the timing of both collecting the patient sample and obtaining samples for establishing a normal range for the laboratory. An increased plasma level of PAI-1 is associated with impaired fibrinolytic function. Elevated levels of PAI-1 have been observed in thrombolytic disease, acute myocardial infarction, DVT, normal pregnancy, sepsis, cardiovascular accident, strokes, type 2 diabetes, DIC, and when the subject's condition is resistant to thrombolytic therapy. Decreased levels can be evident with severe liver disease, amyloidosis, and fibrinolytic therapy regimens.

Tissue Plasminogen Activator (tPA)

Functional (chromogenic) and antigenic (ELISA) assays are available to measure tPA, an important protein in the fibrinolytic system. The physiologic role of tPA is to activate plasminogen to plasmin, which then degrades fibrin to soluble FDPs. Specific interactions between tPA and fibrin and between plasmin and the plasmin inhibitor, α2-antiplasmin, regulate fibrinolysis. In the assay for tPA, PAI-1 is usually present in large excess and must be prevented from quenching tPA activity. This is accomplished using a special blood collection tube (Stabilyte, an acidified citrate tube available from Diagnostica Stago US [formerly TCoag], Parsippany, New Jersey) that provides mild acidification and stabilization of the sample, blocking the effect of PAI-1. Decreased tPA levels have been reported in myocardial infarction, ischemic stroke, and DVT. This marker is an acute phase reactant and can be elevated in subjects with cancer, postoperative surgery, pregnancy, metabolic syndrome, and sepsis.

Thrombin-Activatable Fibrinolysis Inhibitor (TAFI)

TAFI induces hypofibrinolysis by decreasing fibrin's ability to bind tPA and plasminogen. High concentrations of TAFI can induce a hypofibrinolytic state and an elevated risk of thrombosis, whereas low levels of TAFI can be associated with excessive bleeding. Both standard ELISA assays for TAFI antigen and functional assays are now available. The TAFI activity can be assessed by its ability to delay the lysis of a plasma clot induced by recombinant tPA and chromogenic assays. The TAFI antigen concentration in normal human plasma is usually between 40–250%. There are no variations for gender or pregnancy.

☑ CHECKPOINT 36-8

What is the appropriate interpretation of the following coagulation test results?

α2-antiplasmin: Decreased
D-dimer: Elevated
ECL: Prolonged
PAI-1 activity: Increased
Plasminogen activity: Decreased

LABORATORY EVALUATION OF ANTICOAGULANT THERAPY

A critical function of hemostasis testing is to monitor patients on anticoagulant therapy. As with diagnostic testing, quality assurance standards for blood collection, specimen processing, and quality control must be maintained.

Oral Anticoagulant Therapy and the Prothrombin Time—INR Value

When monitoring **oral anticoagulant therapy** (Coumadin, warfarin), the clinical laboratory reports patient values using the **international normalized ratio (INR)**, which is determined from the PT result. The INR is used to standardize the PT results for the various reagents and instruments used for this test. This standardization of oral anticoagulant control has greatly improved patient care in this mobile world.

Each manufacturer has an in-house standard (a specific lot of its own thromboplastin) that has been carefully calibrated against one of the World Health Organization (WHO) standards. The **international sensitivity index (ISI)** value is calculated as a specific correction factor for a manufacturer's standard against the WHO reference preparation. The ISI for a particular reagent is instrument dependent. The reagent manufacturer usually supplies an ISI for the most common reagent/instrument combinations. Its value is listed on each lot number of thromboplastin reagents purchased for PT/INR testing. Commercial manufacturers of recombinant thromboplastin reagents are working to produce ISI values close to 1.0. This value is the WHO-assigned ISI that is recommended in an effort to minimize discrepancies between labs in reporting the INR value.[84,85] Most laboratories currently report both the PT and the INR for a given sample. The advantage of the INR is that it is independent of the reagents and methods used to determine PT and therefore allows better assessment of long-term oral anticoagulant therapy. By definition, the INR is the PT ratio equivalent to using the WHO international reference preparation as the source of thromboplastin in the performance of a PT. Each laboratory uses the formula in Figure 36-6 ■ to calculate each INR value from the PT produced for each patient. The calculation also includes the lab's geometric mean value for at least 20–30 normal plasma control subjects using the specific lot of thromboplastin with a particular type of instrumentation (geometric mean normal PT for the laboratory). The geometric mean value determination should be performed with each new lot of reagent.

By reporting the PT as an INR value, each laboratory is using a standardized unit related to the WHO standards. The INR value has also provided the opportunity to use a common unit for defining the **therapeutic ranges** for oral anticoagulant therapy. Patients receiving oral anticoagulants for a hypercoagulable state (arterial or venous thromboembolism) should have an INR between 2.0 and 3.0; occasionally (e.g., with a mechanical valve replacement), the recommended ratio can be higher. The INR was devised to monitor patients on long-term anticoagulant therapy and is thus not meaningful and should not be reported for patients not on it.

The efficacy of oral anticoagulants such as warfarin and Coumadin is influenced by the patient's body mass, vitamin K content in the diet, interactions of drugs and supplements, wellness, and hepatic function. The INR should be carefully interpreted with regard to the patient's drug regimen (antibiotics or aspirin-containing medication) and dietary changes. POC instruments that determine the individual's INR from a capillary puncture are currently available. The reagent used by these instruments is only thromboplastin; calcium is not required because the blood sample has not been citrated; thus, the calcium is not bound and is available from the patient's whole blood. These instruments determine the PT and calculate the INR. Patients, anticoagulation clinics, and laboratories in physicians' offices use them to monitor and maintain optimal therapeutic levels of oral anticoagulant.

$$INR = R^{ISI}$$

Where R = PT ratio obtained with the working thromboplastin
PT ratio = Patient's PT/Mean normal PT
ISI = International sensitivity index (provided by manufacturer)

Example: Patient's PT = 21.5 seconds
Mean normal PT = 12.0 seconds
ISI = 1.35

$$INR = (21.5/12.0)^{1.35} = 2.2$$

■ **FIGURE 36-6** International normalized ratio (INR) calculation formula.

Heparin Therapy Monitoring

Unfractionated heparin (UFH) and low-molecular-weight heparins (LMWHs) such as enoxaparin, dalteparin, tinzaparin, nordraparin, and fondaparinux, potentiate the activity of antithrombin and covalently neutralize thrombin and activated factor Xa (FXa).[86] Traditionally, the APTT has been the most commonly used procedure to monitor standard UFH therapy. The TT can also be used. If monitoring is necessary for LMWH, the factor Xa inhibition assay is used.

Activated Partial Thromboplastin Time (APTT)

Traditionally, the APTT has been the most commonly used procedure to monitor standard UFH therapy. The accepted procedure is to establish a therapeutic range for UFH. The coagulation laboratory must collect 50 or more specimens from subjects with different levels of UFH present. Samples from patients receiving both UFH and oral anticoagulants are unacceptable. The APTT results and the heparin concentrations (determined by a chromogenic anti-FXa heparin assay) are analyzed by regression analysis to obtain a dose response curve. The APTT therapeutic range is extrapolated at the heparin concentration between 0.3 and 0.7 U/mL on the dose response curve. The College of American Pathologists (CAP) requires that clinical laboratories establish a heparin therapeutic range for their APTT procedure.[87,88] The degree of prolongation of the APTT is variable, depending on the APTT reagent/instrument combination, and even between different lots of the same commercial reagent (Web Figure 36-5). A baseline APTT should be determined before the subject is given the initial UFH bolus. If the APTT is prolonged before heparin administration, a coagulation deficiency or a lupus anticoagulant could be present. If so, the patient should be monitored with the chromogenic anti-FXa assay for heparin therapy. Some very sensitive APTT reagents do not give measurable CTs at heparin concentrations of 0.5 U/mL or higher, thus eliminating their usefulness in monitoring heparin levels.

★ **TABLE 36-8** Weight-Based Heparin Dosing Nomogram

Initial Heparin Dose: Initial Maintenance Infusion Rate		**80 Units/kg Bolus 18 Units/kg/hr**
Adjust maintenance infusion: Perform an activated partial thromboplastin time (APTT) 6 hours after the initial heparin bolus and adjust as follows.		
APTT, sec[a]	**Bolus**	**Change in Infusion Rate of Heparin**
<40	80 units/kg	Increase infusion by 4 units/kg/hr
40–49	40 units/kg	Increase infusion by 2 units/kg/hr
50–80		No change
81–100		Decrease infusion by 2 units/kg/hr
>100		Stop heparin infusion for 1 hour; then decrease infusion by 3 units/kg/hr

An APTT should be performed 6 hours after each dosage change and the appropriate changes in dosage applied.

[a]Based on its established heparin therapeutic range, each laboratory should determine its own APTT ranges for the nomogram.

There currently is no standardization of the APTT to normalize differences in reagents/test systems as with the ISI of the PT reagents. In addition to the sensitivity issues mentioned, complications include the variation in standard heparin preparations, the individuality of a patient's response to heparin from heparin-binding proteins, and the physiologic clearance mechanisms. For these reasons, two individuals receiving a standard heparin bolus of 5000 units in the same facility can have very different APTT results (one indicating a therapeutic response and the other a supratherapeutic response). Thus, the common practice of aiming for an APTT result that is 1.5 to 2.5 times the control APTT value is no longer acceptable.

A weight-based heparin dosing **nomogram** (usually created in conjunction with the pharmacy) should also be provided to the clinicians so that clinicians can determine the appropriate dose of heparin to reach and/or maintain a therapeutic range [89,90] (Table 36-8 ★). In addition, a variety of factors can impact heparin monitoring (Table 36-9 ★).

Some institutions currently monitor all of the heparin therapies, which include UFH and LMWHs with only the chromogenic anti-FXa testing because of the problems with preanalytical variables that affect the APTT.

Thrombin Time

TT is less commonly used to monitor heparin therapy. Its advantage is that it is not influenced by plasma factor deficiencies. It is primarily used to detect the presence of heparin because of its sensitivity in the monitoring of heparin anticoagulation.[91]

Anti-Xa Assay

Low-molecular-weight heparins (LMWH) (Chapter 33) have become a common form of heparin for anticoagulant therapy. It has more reliable **pharmacokinetics** and bioavailability than standard heparin and thus does not require routine laboratory monitoring in adults because its dose response is more predictable. Monitoring should be performed on obstetric patients who have a history of spontaneous abortion, morbidly obese patients, subjects who are severely underweight, neonates, or individuals with renal issues. The FXa inhibition assay (anti-Xa assay) is recommended if monitoring is necessary.

Therapeutic doses of LMWH are based on an anti-FXa level of 0.5 to 1.0 units/mL. A citrated blood sample is taken 4 to 6 hours after a subcutaneous injection. A standard for performing the anti-FXa assay for LMWH is used. The anti-Xa assay is also a more specific test for monitoring unfractionated heparin (UFH) therapy. A special standard is used to test for UFH.

The **chromogenic assay** for both types of heparin uses excess activated FX (FXa), which is added to the patient's PPP and incubated.[86,92] Heparin with antithrombin present in the patient's PPP inhibits the FXa. A chromogenic substrate is added to this mixture, and any residual FXa enzymatically cleaves the chromogen, producing a yellow color that is measured spectrophotometrically. A reference (standard)

★ **TABLE 36-9** Factors Affecting the Monitoring of Heparin

Conditions that influence the pharmacokinetics or general bioavailability of heparin: Heparin binds to various plasma proteins (e.g., platelet factor 4, fibrinogen, VWF, fibronectin, and vitronectin), macrophages, and endothelial cells, resulting in decreased bioavailability

Conditions that alter the characteristics of the activated partial thromboplastin time dose response to heparin

Conditions that cause an abnormal baseline activated partial thromboplastin time (factor deficiency, lupus anticoagulant)

Patient's body weight

Antithrombin level

Specimen collection and processing problems

- Time of collection (minimum of 4 hours needed to achieve steady state with heparin)
- Delayed testing (can result in abnormally prolonged results)
- Failure to achieve platelet-poor plasma (can result in release of PF4 from activation of residual platelets resulting in underestimation of heparin in the sample)

curve is prepared by plotting the various dilutions of known concentrations of heparin against its corresponding absorbance. The results for the patients and controls are obtained from this curve using the respective absorbance readings. The therapeutic range for heparin is 0.3–0.7 U/mL.

☑ CHECKPOINT 36-9

As seen in Figure 36-6, the patient has a PT of 21.5 seconds (producing an INR of 2.2). In this case, the patient is not on oral coagulation. What is the explanation for not reporting the INR?

MOLECULAR MARKERS OF HEMOSTATIC ACTIVATION

A more detailed understanding of the biochemistry of coagulation and fibrinolysis has resulted in the development of a number of sensitive and specific assays that detect molecular markers of platelet activation, generation of coagulation enzymes, and products of intravascular fibrin formation or dissolution (Table 36-10 ★). The availability of testing for molecular markers is useful for the early detection of platelet-driven or coagulation-driven disorders and their use can result in improved medical care for thrombotic and fibrinolytic disease. This testing is not, however, routine and could be available only at large medical centers or reference laboratories.

Markers of Fibrin Formation and Fibrinolysis

The process of activating coagulation converts a number of zymogens to active serine proteases. Direct measurements of the levels of most hemostatic enzymes in vivo (e.g., thrombin, plasmin) are not possible because naturally occurring protease inhibitors rapidly neutralize the majority of them. However, the activation peptides released on activation of the zymogen can be measured by immunochemical assays (radioimmunoassay, ELISA). The enzyme-inhibitor complex that forms as a result of zymogen activation can also be measured by similar immunochemical methods. Because of the high cost and low volume of test requests, the routine clinical laboratory generally does not offer these assays.

The immunochemical assays depend on the development of monoclonal or polyclonal antibodies capable of recognizing antigenic determinants on the activation fragment or enzyme-inhibitor complex that are hidden in the parent zymogen or inhibitor. More widely used assays include those for: fibrinopeptide A and/or fibrinopeptide B and prothrombin activation fragment 1.2. Thrombin-antithrombin (TAT) complex and PAP complex testing were described earlier in the chapter.

★ **TABLE 36-10** Immunochemical Markers of Hemostatic Activation

Coagulation	
Activation peptides	Factor IX activation peptide
	Factor X activation peptide
	Prothrombin activation fragment F1 + 2
	Protein C activation peptide
	Fibrinopeptide A, fibrinopeptide B
Enzyme-inhibitor complexes	Thrombin-antithrombin complex (TAT)
	Factor Xa-antithrombin complex
	Factor IXa-antithrombin complex
	Activated protein C-protein C inhibitor complex
Fibrinolysis	Bβ1–42 fragment (plasmin cleavage of fibrin)
	Plasmin–antiplasmin complex (PAP)
Platelet activation (flow cytometry)	Activated GPIIb/IIIa complex
	P-selectin (CD62-P)
	GPIb/IX/V (decrease)

Laboratory Markers of Platelet Activation

Platelet hyper-reactivity or circulating activated platelets have been reported to be associated with several clinical conditions including coronary artery disease, unstable angina, and acute myocardial infarction. The bleeding time, platelet function analyzer, and platelet aggregation tests remain the only standard clinical tests of platelet activation and function, but they are more useful in adults than pediatric patients in assessing platelet hyper-reactivity (see earlier section "Laboratory Investigation of Primary Hemostasis"). A number of additional tests have been developed and are available in specialized coagulation laboratories, including ELISA assays for PF4, βTG, and soluble P-selectin (markers of platelet activation and secretion), and urine assays for thromboxane A_2 metabolites ($TX\text{-}B_2$).[93]

Flow cytometry can be used to evaluate platelet activation and function and likely assumes an increasingly important role in the evaluation of in vivo platelet activation. Platelets are labeled with a fluorescent-conjugated antibody, and specific characteristics of a large number of individual platelets can be measured rapidly (1000–10,000 cells/minute). In the absence of an added (exogenous) platelet agonist, whole blood flow cytometry evaluates the activation state of circulating platelets according to the binding of an activation-dependent monoclonal antibody. If an exogenous agonist is included in the assay, it is possible to analyze the reactivity of circulating platelets in vitro (i.e., a physiologic assay of platelet function).

Monoclonal antibodies can be used to measure the expression of platelet surface antigens. However, antibodies that detect activation-dependent antigens (i.e., antibodies that bind to activated platelets, not to resting platelets) are particularly useful for assessing platelet hyper-reactivity. Monoclonal antibodies that detect an activation-induced conformation change in GPIIb/IIIa appear to be directed against the fibrinogen-binding site of GPIIb/IIIa of activated platelets. P-selectin (CD62-P) (previously referred to as *GMP-140* or *PADGEM*) is a component of the α-granule membrane of resting platelets and is expressed on the platelet surface after α-granule secretion. Thus, a P-selectin specific antibody binds only to degranulated, activated platelets, not to resting platelets.

The GPIb/IX/V complex (VWF receptor) offers a different approach in evaluating in vivo platelet function. In contrast to

activation-dependent monoclonal antibodies (e.g., GPIIb/IIIa), the binding of GPIb/IX/V–specific antibodies to activated platelets is markedly decreased compared with that of resting platelets. This decrease occurs because of a redistribution of the GPIb/IX/V complex to the membrane of the surface-connected canalicular system with platelet activation. Specific testing for these platelet markers is available only in a large clinical flow cytometry laboratory or a university setting.

Evidence of in vivo platelet activation can have significant clinical implications in cardiovascular and thrombotic diseases. Flow cytometric analysis of platelet activation-dependent markers can be used to determine optimal antiplatelet therapy in clinical settings and to measure platelet hyper-reactivity in a number of clinical conditions.

GLOBAL TESTING

POC hemostasis testing has become standard in the majority of hospitals. Increasing numbers of complex surgical procedures (liver transplantation, coronary bypass graft, valve repair in patients on the heart-lung machine) have created a demand for the availability of rapid and more comprehensive information on a patient's hemostatic condition. Therapeutic decisions must be made quickly to discriminate between surgical bleeding and a hemostatic disorder.

Typical coagulation tests (PT, APTT, TT) artificially compartmentalize hemostasis (e.g., intrinsic, extrinsic pathways). They measure only the initiation of clotting (initial thrombin dependent conversion of fibrinogen to fibrin) and not its speed or total extent. They are performed in the absence of cellular elements and provide data only on plasma clotting components, thereby overlooking potentially important interactions essential to the clinical evaluation of in vivo hemostasis. In contrast, **global testing** instruments analyze the entire hemostasis process including coagulation, anticoagulant effects, fibrin formation and stabilization, clot retraction (platelet function), and fibrinolysis.[94]

The first two instruments described are primarily found in surgery settings where blood product usage can be monitored and in research facilities to improve diagnostic testing and monitor treatment of patients with bleeding and clotting disorders.

Thromboelastography (TEG)

TEG, a viscoelastic whole blood instrument introduced by Hartert in 1948, is increasingly being used as a near-patient testing method for assessing blood coagulability. The instrumentation (thromboelastograph) has become more clinically useful with direct readout of results and shortened reaction times by using activators and inhibitors in the cuvettes. The TEG evaluates clot formation from initiation to breakdown, and provides prompt, reliable information to clinicians on the causes of blood loss in critical POC situations. It allows the discrimination between surgical bleeding and hemostasis disorders. The TEG seems to be the most suitable method for the detection of hyperfibrinolysis, an otherwise difficult to analyze coagulopathy that can lead to massive bleeding (Web Figures 36-6, 36-7, 36-8).

The TEG now is able also to assess platelet function in patients who received platelet-inhibiting drugs such as aspirin, clopidogrel, and others. The specialized testing is called *TEG® platelet mapping assay*.

ROTEM®

The ROTEM® Haemostasis Whole Blood Analyser is a computerized device based on the TEG. It is designed to be used with appropriate reagents to generate both a qualitative graph and quantitative numerical results. The standardized reagent menu allows for the differentiation of coagulation factor deficiencies, anticoagulant effects, platelet disorders, fibrin polymerization disorders, and hyperfibrinolysis (Web Figures 36-9 and 36-10).

Calibrated Automated Thrombogram (CAT)

Thrombin participates in a series of positive and negative feedback reactions that first increases its own production significantly but later inhibits it. Therefore, the way in which thrombin is formed and then decreases when blood coagulation is triggered is an important indicator of the overall function of the hemostatic system. The thrombin generation test (TGT) was developed as an easy way to monitor the rise and fall of thrombin in clotting plasma. The calibrated automated thrombogram (CAT) is a TGT that can measure both hypercoagulability and hypocoagulability and is sensitive to the action of most antithrombotic drugs. In the CAT, the test wells are filled with reagents consisting of thrombin calibrator in one set of wells and a trigger necessary to start coagulation (either PPP or PRP reagent) in another set of wells. After the addition of plasma, the instrument dispenses a mixture of fluorogenic substrate and calcium so that coagulation begins. The analysis program calculates all parameters of the thrombogram (including endogenous thrombin potential, peak height, lag time, and more) and expresses the results in nanomoles of thrombin over time.[95]

☑ CHECKPOINT 36-10

A patient has a venous thrombotic episode and is to be treated appropriately. When should the blood sample be drawn to perform a thrombotic workup to diagnose an acquired or congenital deficiency? Why?

HEMOSTASIS INSTRUMENTATION

Hemostasis testing has advanced from inefficient manual methods to fast, accurate, and fully-automated analyzers. The systems have been improved to save money, reduce maintenance requirements, and increase productivity in the clinical hemostasis laboratory.

Evolution of Hemostasis Testing

Three centuries ago, clot detection in animal and human blood was checked only by the human eyes. With the invention of the microscope in the 1800s, scientists could examine the blood closer for changes in turbidity and fibrin formation. Temperature control, resistance

monitoring by change in voltage, and the use of glass capillary tubes to view clot formation further enhanced hemostasis testing from 1822 to 1922. In the early 1900s, researchers measured the time it took whole blood to clot while being tilted in tubes. This was the precursor to the Lee-White tilt tube procedure in subsequent years.[96] The coagulometer developed by Kottman in 1910 determined CTs by measuring the change in whole blood viscosity as it clotted. The change in viscosity was determined by plotting the voltage against time. Plasma-based coagulation testing can be traced back to 1920. During this era, the technique of adding calcium chloride to anticoagulated plasma at 37°C and measuring the increasing viscosity of the plasma during fibrin monomer polymerization began. This led to development of the PT and PTT. TEG and sonar clot detection assays are also based on this principle.

Kugelmass introduced a new method of clot detection in 1922. He adapted the nephelometer to coagulation testing. **Nephelometry** measures scattered light at an angle or records the change in light transmission. Fibrin disperses light in forward (180°) and side angles (90°). When the amount of scattered light or transmitted light reaches a specific predetermined intensity, the timer stops. The instrument measures the light scattered or transmitted before and after the clot formation. The difference between the two measurements is proportional to the amount of fibrin formed.[97]

Quick developed the original method for measuring the PT in 1935. He mixed thromboplastin (extracted from human brain) with plasma, added calcium ions, and observed the clot formation at 37°C. Under these conditions. Quick found that fibrin clots usually developed within 12 seconds.[98] In 1936, Baldes and Nygaard introduced the photoelectric method for clot detection. They were able to determine CTs by measuring increased optical density during clot formation in plasma samples.

By the 1960s, the fibrometer and other electromechanical semiautomatic instruments began replacing manual methods in laboratories. The fibrometer is considered the prototype for the electromechanical instruments. It provided temperature control and included a pair of electrodes (one moving, one stationary) and a timing device to detect clots at 0.5-second intervals. The moving electrode cycles through the reaction mixture in an elliptical pattern created by raising and lowering the probe foot. The moving then becomes electrically active when it is above the reaction mixture. As the fibrin clot is formed, the small hook on the tip of the moving electrode picks it up. The electric circuit is completed when the moving electrode becomes activated as it moves out of the reaction mixture; current flows from the stationary electrode through the reaction mixture and fibrin clot to the moving electrode. The creation of the electric circuit causes the timing device to stop. Other electromechanical systems such as the Diagnostica Stago ST-4® instrument measures CTs in a cuvette containing a rolling steel ball in an electromagnetic field.[99] This instrument is described in the section "Electromechanical Clot Detection Systems."

In 2000, the CAP coagulation proficiency survey indicated that approximately 25 different instruments are being used for hemostasis testing.[100] Many of the mechanical and optical clot detection instruments have been enhanced to include chromogenic and immunologic methodologies. The AMAX Destiny Plus is an analyzer with the mechanical and optical clot detection method on one platform.

Automated Hemostasis Analyzer Methodologies

The methodologies available today include electromechanical, photo-optical, chromogenic, and immunologic. Some instruments use a combination of these principles.

Electromechanical Clot Detection Systems

Most laboratories have replaced the fibrometer with more advanced electromechanical instrumentation. Diagnostica Stago hemostasis analyzers utilize innovative electromechanical systems. They are viscosity-based detection methods. The instruments monitor the motion (amplitude) of a steel ball inside a cuvette in a magnetic field as the viscosity of the plasma changes during clot formation. As the clot appears, the viscosity increases and the amplitude of the ball motion decreases, the timer stops at algorithmic predetermined times.[99] Electromechanical clot detection systems are insensitive to colored and hemolyzed plasmas, lipemic plasmas, bilirubin, and turbid reagents.

Optical/Photometric/Turbidimetric Clot Detection Systems

Photometric and/or optical (photo-optical) hemostasis analyzers measure the increase in turbidity when fibrinogen is converted to insoluble fibrin. Reagent and plasma are automatically pipetted into sample cups or cuvettes. After precise mixing and incubation phases, the system measures the change in plasma OD and measures the end point of clot formation. Many of the optical systems have filters and OD correction factors to minimize the effects of markedly lipemic and icteric plasma samples. In 2012, Beckman Coulter Inc./Instrument Laboratory released the ACL TOP CTS, which uses LED technology. The 671-nm wavelength range of the LED light allows the instrument to significantly eliminate the lipemia, hemolysis, and bilirubin preanalytical variables. Siemens Healthcare Diagnostics Sysmex CAs and BCS analyzers utilize optical/**turbidimetric clot detection** methods.[100]

Nephelometry was originally used for immunoassays. The antigen–antibody complexes precipitate resulting in turbidity, which scatters light. In reactions when the immune complexes are too small for detection, the antibodies are first attached to microlatex particles. When the nephelometry detection method is used for coagulation immunoassays, specific factor antibodies are bound to latex particles. This method provides quantitative but not functional coagulation factor assays on complex automated hemostasis analyzers. The nephelometry technology is also used to produce clot signature curve analyses (continuous readings of the clotting sequence).

Chromogenic Detection Methods

Chromogenic methods are based on the use of a chromophore (color-specific generating substance) such as para-nitroaniline (pNA), which has a maximum absorbance of 405 nm (Web Figure 36-11a). In chromogenic testing, the pNA is conjugated to a colorless, synthetic oligopeptide substrate that mimics the target sequence of the activated coagulation factor being measured (Web Figure 36-11b). Coagulation

protease cleaves the chromogenic substrate at a specific binding site in the amino acid sequence of the oligopeptide. The pNA is released, and a photodetector measures the OD at 405 nm. Free pNA is yellow, and the intensity of the color is proportional to the amount of pNA released. Depending on the assay's sequence of reactions, the change in OD can also be indirectly proportional to the activity of the analyte, for example in the antifactor Xa assay. Fluorogenic methods (e.g., the calibrated automated thrombogram) for hemostasis testing use a fluorescent substrate.

Immunologic Detection Methods

Latex microparticles are coated with a specific antibody against the antigen being determined. A monochromatic light beam passes through the latex microparticle suspension. The light wavelength is larger than the diameter of the suspension microparticles. Therefore, the microparticles absorb only a small amount of light. However, when the suspension of latex microparticles is mixed with test plasma containing the antigen, they adhere to the antibody forming agglutinates. An increase in agglutination or turbidity results in increased light absorbance. The antigen level is proportional to the amount of light absorbed. A standard calibration curve is used to determine the actual level. The Diagnostica Stago STA-Liatest® VWF:Ag assay is an example of a immunoturbidimetric method.[100]

Selecting a Hemostasis Analyzer

Automated hemostasis analyzers are available for laboratories performing routine or specialized testing and test volumes that are low, medium, or high. The coagulation market is very competitive with customers continuously looking to do more with current analyzers. Manufacturers must keep up with the customers' demands for more reliable, accurate, user-friendly, cost-effective and safe instrumentation. Some of the features to consider when comparing or selecting a hemostasis analyzer are the following:[97]

- *Methodologies* The type of clot detection system (optical or mechanical) and the other test methods (chromogenic, immunologic) the instrument supports.
- *Random access testing* The instrument's capability to perform multiple tests on the same or different samples in any order.
- *Reagent systems* The instrument's capability to accept alternative manufacturers' reagents for the same methodology.
- *Level sensing* The instrument's capability to verify reagent volumes, monitor sample aspirations, and alert the operator if volumes are inadequate.
- *Barcode reading* The instrument's capability to read barcodes on samples and reagents for positive identification such as name, accession or lot number, and other information.
- *Tube sampling* The instrument's capability to perform closed-tube sampling (penetration of the cap) for safe handling or open-tube (cap removed) sampling.
- *Computer and software features* The capability of the instrument's computer to interface with the laboratory's information system; store and retrieve patient data; manage quality control data; add and manage test protocols; program reflex testing algorithms; provide program flags or alerts for specimen integrity (e.g., lipemia or hemolysis), instrument malfunctions, quality control failures, and critical values.
- *Reflex testing* The programmability of the instrument's software to repeat or add tests under defined conditions set by the operator.
- *Throughput* The number of tests the instrument can accept and process within a given time period.
- *STAT processing* The instrument's capability and the time it takes to process high-priority tests during operation.
- *Cost savings* The instrument's capability to perform tests with reduced reagents and smaller sample volumes and to operate with lower consumable costs.
- *Downtime* The amount of time the instrument requires for scheduled preventive maintenance procedures and unexpected repairs (check the distributor's technical support and service availability).
- *Footprint* The amount of space the benchtop or floor model instrument requires.
- *Training and support* The company's training program and technical support for the instrument.

Point-of-Care (POC) Hemostasis Instrumentation

Point-of-care (POC) instruments and bedside or self-monitoring hemostasis testing systems provide a convenient method to monitor oral anticoagulant therapy. Most of these POC instruments are portable or handheld, requiring as little as 10 mcL or up to 2 mL of whole or anticoagulated blood. Some features and functions available on POC hemostasis instruments include the following:

- Multiple specimen types
- Integrated quality control with each analysis
- Automatic quality control lockout
- Onboard system for automatic error detection

Previous sections of this chapter described several other hemostasis instruments for platelet function testing and other assays.

Summary

This chapter reviewed the laboratory tests performed in the coagulation laboratory and specialized tests available from research or reference laboratories. It highlighted the role of the laboratory professional as an integral part in the diagnosis of bleeding and clotting disorders. The additional technical information provided on this chapter's Companion Resources allows the MLS/MLT to access information to improve coagulation testing so that the knowledge of all intricacies of sample collection, processing, quality control, accuracy, and reproducibility are available for each test.

The tests described are grouped according to the part of the hemostatic system that they assess: primary hemostasis, secondary hemostasis, fibrinolysis, and hypercoagulable states. A combination of tests from these groups can be selected to screen for a disorder of the hemostatic system. As an example, the PFA, PT, and APTT can be used to identify a defect in either primary or secondary hemostasis. When this defect has been identified, specific laboratory tests can be selected to provide information for making a definitive diagnosis of the coagulation disorder. Based on the results of screening tests for fibrinolysis (TT, D-dimer), further fibrinolytic testing can be performed. Laboratory tests used to evaluate hypercoagulable states (e.g., AT, PC, PS, APCR) and to monitor anticoagulant therapy (e.g., PT/INR, APTT, FXa inhibition assay) are some of the most frequently ordered in the coagulation laboratory. Testing is now available to detect activated platelets, coagulation enzymes, and products of intravascular fibrin formation or dissolution. Global testing once used only in the research setting is becoming clinically available. Manual testing methods have been replaced by electromechanical, photo-optical, chromogenic, and immunologic methods.

Review Questions

Level I

1. The hematology laboratory informs you that the hematocrit on a patient is 57% (0.57 L/L). For the proper anticoagulation of a 5.0 mL sample of the patient's blood, how much 3.2% sodium citrate should be used? (Objective 1)

   A. 0.22 mL

   B. 0.40 mL

   C. 0.57 mL

   D. 0.96 mL

2. What laboratory test is used to monitor oral anticoagulant therapy? (Objective 4)

   A. FXa inhibition assay

   B. bleeding time

   C. PT/INR

   D. APTT

3. What is the specimen of choice for the PT and APTT procedures? (Objective 3)

   A. platelet-rich plasma, citrated

   B. PPP, citrated

   C. serum

   D. plasma, heparinized

4. A physician asks you to recommend a coagulation test for secondary fibrinolysis. Which laboratory test is specific for fibrinolysis? (Objective 2)

   A. D-dimer

   B. FDPs

   C. TT

   D. antithrombin

5. Which of the following reagents is used to determine fibrinogen concentration? (Objective 3)

   A. thromboplastin

   B. reptilase

   C. thrombin

   D. ellagic acid

6. Calculate the INR using the following data: (Objective 6)

Patient's PT	23.5 sec
Mean normal PT	11.5 sec
ISI	1.15

   A. 2.0

   B. 2.3

   C. 2.5

   D. 1.7

7. The APTT is a screening test for the laboratory evaluation of inherited and acquired deficiencies in which of the following? (Objective 2)

   A. extrinsic pathway of the coagulation cascade

   B. intrinsic pathway of the coagulation cascade

   C. platelets

   D. vascular system

8. The combination of a prolonged APTT and a prolonged APTT with the mixing study procedure indicates the presence of what? (Objectives 2, 7)

   A. circulating inhibitor

   B. FVIII deficiency

   C. antiplatelet antibodies

   D. excessive vitamin K

9. What is the most likely factor deficiency based on the following data? (Objective 7)

PT	Normal
APTT	Prolonged
TT	Normal

A. FVII

B. FII

C. FIX

D. FXIII

10. Which laboratory test evaluates primary hemostasis? (Objective 2)

A. PFA-100®

B. reptilase time

C. PT

D. protein C assay

11. A coagulation testing instrument that detects a decrease in motion of a rolling steel ball when a clot forms is an example of which technology? (Objective 9)

A. chromogenic

B. immunologic

C. electromechanical

D. photometric

12. An advantage of the coagulation analyzers that use the electromechanical technology is that they: (Objective 9)

A. are more sensitive

B. are not affected by colored or hemolyzed plasma

C. do not need calibration

D. use a smaller sample size

13. Which is *not* a feature of a fully automated hemostasis analyzer? (Objectives 9, 10)

A. reagent volume flagging

B. sample dilutions

C. reagent barcode reading

D. specimen centrifugation

14. Hemostasis analyzers support the following assays *except*: (Objective 10)

A. potientiometric

B. immunologic

C. chromogenic

D. turbidimetric

Level II

1. Which laboratory test is used to investigate the hypercoagulable states? (Objectives 3, 5)

A. thrombin time

B. plasminogen

C. APCR

D. euglobulin lysis

2. Platelet aggregation studies revealed normal aggregation curves with collagen, epinephrine, and ADP but an abnormal aggregation curve with ristocetin. Based on these findings, what is the differential diagnosis? (Objective 5)

A. VWD and BSS

B. Glanzmann's thrombasthenia and VWD

C. storage pool disease and Glanzmann's thrombasthenia

D. BSS and storage pool disease

3. The observation of a normal reptilase time and a prolonged thrombin time indicates which disorder? (Objective 5)

A. presence of fibrin degradation products

B. dysfibrinogenemia

C. hypoplasminogenemia

D. presence of heparin

4. The activated PC resistance assay on a specimen gave the following results:

Standard APTT	28.4 sec
Modified APTT with APC	71.6 sec

How would you interpret this assay? (Objective 5)

A. presence of factor V Leiden

B. increased levels of activated FVIII

C. presence of normal FV

D. decreased levels of antithrombin

5. Given the following laboratory results, what is the appropriate reflex test? (Objective 6)

PT	Normal
APTT	Slightly prolonged
Platelet aggregation studies	
Collagen	Normal
ADP	Normal
Ristocetin	Abnormal

A. ristocetin cofactor assay

B. D-dimer

C. PK screen

D. TT

6. What is the most likely cause of the following laboratory data? (Objective 5)

PT	Normal
APTT	Prolonged
TT	Normal
APTT with 10-minute incubation	Normal

   A. presence of lupuslike anticoagulant
   B. FXII deficiency
   C. increased levels of tissue plasminogen activator
   D. PK deficiency

7. Which of the following conditions does *not* have an effect on heparin monitoring using the anti-Xa assay? (Objective 7)

   A. patient's body weight
   B. delay in testing
   C. use of citrated PPP
   D. underlying liver disease

8. In monitoring a patient on oral anticoagulant therapy, an INR of 1.3 was obtained. How would you interpret this result? (Objective 7)

   A. The patient is adequately anticoagulated and should be tested again in 1 month.
   B. The patient is underanticoagulated and should be evaluated for a change in dietary habits.
   C. The patient is overanticoagulated and should receive a vitamin K injection.
   D. Data to determine the patient's status are insufficient.

9. Based on the following laboratory data, which is the appropriate reflex test? (Objective 6)

PT	Normal
APTT	Prolonged
TT	Normal
Mixing studies	Correction

   A. dRVVT
   B. FXI assay
   C. APCR assay
   D. FDP assay

10. Given the following laboratory data, which assay would you perform next to resolve these results? (Objective 6)

PT	Prolonged
APTT	Prolonged
TT	Prolonged
Mixing studies	No correction

   A. a reptilase time to confirm heparin contamination
   B. a fibrinogen determination to confirm fibrinogen deficiency
   C. a dRVVT to confirm lupuslike anticoagulant
   D. a D-dimer test to confirm consumptive coagulopathy

11. A safety feature that should be considered when evaluating coagulation analyzers is: (Objective 11)

   A. closed tube sampling
   B. automated disposal of samples
   C. use of disposable tubing
   D. random access sampling

Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

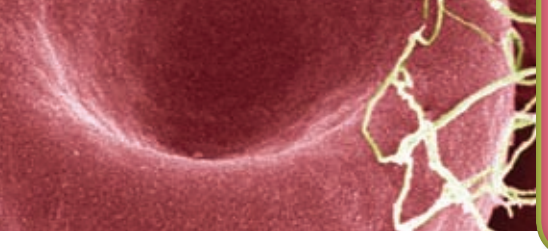
The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Adcock DM, Kressin DC, Marlar, RA. Are discard tubes necessary in coagulation studies? *Lab Med.* 1997;28:530–33.
2. Arkin CF, Davis BH. *Collection, Transport and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays,* 5th ed. Wayne, PA: Clinical and Laboratory Standards Institute. 2008. Approved CLSI Guideline H21-A5.
3. Kottke-Marchant K. An algorithmic approach to hemostasis testing. *Diagn Cytopathol.* 2010;38:461–62.
4. Dade Hepzyme package insert. Deerfield, IL: Dade International Inc./IBEX Technologies; December 1997.
5. Adcock DM, Kressin DC, Marlar, RA. The effect of 3.2% vs 3.8% sodium citrate concentration on routine coagulation assays. *Am J Clin Pathol.* 1997;107:105–10.
6. Scharf RE. Drugs that affect platelet function. *Semin Thromb Hemost.* 2012;38(8):865–83.
7. Burns ER, Lawrence C. Bleeding time: a guide to its diagnostic and clinical unity. *Arch Pathol Lab Med.* 1989;113:1219–224.
8. Gerwirtz AS, Miller ML, Keys TF. The clinical usefulness of the preoperative bleeding time. *Arch Pathol Lab Med.* 1996;120:353–56.
9. Peterson P, Hayes TE, Arkin CF et al. The preoperative bleeding time test lacks clinical benefit: College of American Pathologists' and American Society of Clinical Pathologists' Position Article. *Arch Surg.* 1998;133:134–39.
10. Mielke CH. International committee communications: measurement of the bleeding time. *Thromb Haemost.* 1984;52:210.
11. Siemens Healthcare Diagnostics. Dade-Behring Dade PFA-100® reagents. *To Aid in the Detection of Platelet Dysfunction in Citrated Human Whole Blood.* 2007; B4170 G20AU5734 (97) H/CS/R:5.
12. Christie DJ, Avari T, Carrington LR et al. *Platelet Function Testing by Aggregometry.* Wayne, PA; Clinical and Laboratory Standards Institute; 2008. Approved Guideline CLSI document H-58A.

13. McGlasson DL, Fritsma GA. Whole blood aggregometry and platelet function testing. *Semin Thromb Hemost*. 2009;35:168–80.
14. McGlasson DL, Fritsma GA, Shah AD. Ability of a rapid assay to detect clopidogrel-induced ADP receptor blockade in pre-angiocath subjects. *Blood Coagul Fibrinolysis*. 2011;22:1–5.
15. Favaloro EJ. Clinical utility of the PFA-100. *Semin Thromb Hemost*. 2008;34(8):709–33.
16. McGlasson DL, Fritsma GA. Comparison of four laboratory methods to assess aspirin sensitivity. *Blood Coagul Fibrinolysis*. 2008;19:120–23.
17. Michelson AD, Frelinger AL, Furman MI. Current options in platelet function. *Am J Cardiol*. 2006;98:4N–10N.
18. Weiss HJ. Platelet physiology and abnormalities of platelet function. *N Eng J Med*. 1975;293:531.
19. Mammen EF. Sticky platelet syndrome. *Semin Thromb Hemost*. 1999; 25(4):361–65.
20. Warrier I, Nigro N, Hillman C et al. Platelet activation with stroke and migraine in children. *Thromb Haemost*. 1991;65(6):351.
21. Hilllman CR, Lusher JM, Barnhart MI. Tests of platelet function: technical points of clinical relevance. In: Lusher JM, Barnhart M, eds. *Acquired Bleeding Disorders in Children*. Vol. 4. New York: Masson; 1981.
22. Harms C. Routine laboratory procedures. In: Triplett DA, ed. *Platelet Function, Laboratory Evaluation and Clinical Application*. Chicago: American Society of Clinical Pathology; 1978.
23. Mueller T, Dieplinger B, Poelz W et al. Utility of the PFA-100 and the novel multiplate analyzer for the assessment of aspirin and clopidogrel effect on platelet function of patients with cardiovascular disease. *Clin Appl Thromb Hemost*. 2009;15:652–59.
24. Fritsma GF. Monitoring antithrombotic therapies. In: Rodak BF, Fritsma GA, Keohane EM, eds. *Hematology: Clinical Principles and Applications*, 4th ed. 2012, Elsevier, St. Louis, MO.
25. Santoro SA, Eby CS. Laboratory evaluation of hemostatic disorders. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology: Basic Principles and Procedure*, 3rd ed. Philadelphia: Churchill Livingstone, 2000:1841–50.
26. Sibbing D, Byrne B, Kastrati A. High platelet reactivity and clinical outcome-fact and fiction. *Thromb Haemost*. 2011;106:1-12.
27. Wilson DB. Acquired platelet defects. In: Nathan DG, Ginsburg D, Look AT et al., eds. *Nathan and Oski's Hematology of Infancy and Childhood*, 6th ed. Philadelphia: W. B. Saunders; 2003;1597–1630.
28. Clinical and Laboratory Standards Institute. *One-Stage Prothrombin Time (PT) Test and Activated Partial Thromboplastin (APTT) Test; Approved Guideline*, 2nd ed. Wayne, Penn, CLSI. 2008. CLSI Document H47-A2,
29. Hillman CRL, Lusher JM. Determining the sensitivity of coagulation screening reagents: a simplified method. *J Lab Med*. 1982; 13:162–65.
30. Hathaway WE, Assmus SL, Montgomery RR et al. Activated partial thromboplastins time and minor coagulopathies. *Am J Clin Pathol*. 1979;71:22–25.
31. Marlar RA, Bauer PJ, Endres-Brooks JL et al. Comparison of the sensitivity of commercial PTT reagents in the detection of mild coagulopathies. *Am J Clin Pathol*. 1984;82:436–39.
32. Brandt JT, Arkin CF, Bovill EG et al. Evaluation of PTT reagent sensitivity to factor IX and factor IX assay performance. Results from the College of American Pathologists Survey Program. *Arch Pathol Lab Med*. 1990;114:135–41.
33. Kolde HJ. *Haemostasis, Physiology, Pathology Diagnostics*. Basel, Switzerland: Pentapharm; 2004.
34. Clinical Laboratory and Standards Institute. *Procedure for the determination of fibrinogen in plasma: Approved Guideline*, 2nd ed. Wayne, PA: CLSI. 2001. CLSI Document H30-A2.
35. Ledford-Kraemer MR. Laboratory testing for lupus anticoagulants: pre-examination variables, mixing studies, and diagnostic criteria. *Semin Thromb Hemost*. 2008;34:380–88.
36. Arkin CF, Bovill EG, Brandt JT et al. Factors affecting the performance of factor VIII coagulant activity assays: results of proficiency surveys of the College of American Pathologists. *Arch Pathol Lab Med*. 1992;116:908–15..
37. National Committee for Clinical Laboratory Standards. *Determination of Factor Coagulant Activities*. Villanova, PA: NCCLS; 1997. H48-A.
38. McGlasson DL, Romick BG, Rubal BJ. Comparison of a chromogenic factor X assay with international normalized ratio for monitoring oral anticoagulation therapy. *Blood Coagu Fibrinolysis*. 2008;19:513–517.
39. McGlasson DL, Shaklee PN. A multiinstrument evaluation of a commercial chromogenic factor X assay to monitor oral anticoagulation therapy. *Blood*. 2004;104:4059.
40. McGlasson DL, Fritsma GA. Comparison of two chromogenic FVIII activity assays to a standard clot-based FVIII activity assay. *J Thromb Haemost*. 2009; 7 (supp 2). Abstract PP-WE-235.
41. Triplett DA, Harms, CS. *Prekallikrein (Fletcher Factor) confirmatory test. Procedures for the Coagulation Laboratory*. Chicago: American Society of Clinical Pathology; 1981.
42. Triplett DA, Harms CS. Prekallikrein (Fletcher Factor) synthetic chromogenic substrate assay. *Procedures for the Coagulation Laboratory*. Chicago: American Society of Clinical Pathology; 1981.
43. Triplett DA, Harms, CS. FXIII. *Procedures for the Coagulation Laboratory*. Chicago: American Society of Clinical Pathology; 1981.
44. Jenning I, Kitchen S, Woods TAL et al. Problems relating to the laboratory diagnosis of factor XIII deficiency: a UKNEQAS study. *Thomb Haemost*. 2003;1:2603–608.
45. Quiroga T, Goycoolea M, Munoz B et al. Template bleeding time and PFA-100 have low sensitivity to screen patients with hereditary mucocutaneous hemorrhages: comparative study in 148 patients. *Thromb Haemost*. 2004;2(6):892–98.
46. Dean JA, Blanchette VS, Carcao MD et al. von Willebrand disease in a pediatric-based population—comparison of type 1 diagnostic criteria and use of the PFA-100® and a von Willebrand factor/collagen-binding assay. *Thromb Haemost*. 2000;84(3):401–9.
47. Posan E, McBane RD, Grill DE et al. Comparison of PFA-100 testing and bleeding time for detecting platelet hypofunction and von Willebrand disease in clinical practice. *Thromb Haemost*. 2003;90(3):483–90.
48. Schlammadinger A, Kerenyi A, Muszbek L et al. Comparison of the O'Brien filter test and the PFA-100 platelet analyzer in the laboratory diagnosis of von Willebrand's disease. *Thromb Haemost*. 2000;84(1):88–92.
49. Cariappa R, Wilhite TR, Parvin CA et al. Comparison of PFA-100 and bleeding time testing in pediatric patients with suspected hemorrhagic problems. *J Pediatr Hematol Oncol*. 2003;25(6):474–79.
50. Abildgaard CF, Suzuki Z, Harrison J et al. Serial studies in von Willebrand's disease: variability versus "variants." *Blood*. 1980;56(4):712–16.
51. Sousa NC, Anicchino-Bizzacci JM, Locatelli MF et al. *Haematologica*. 2009;92(2):236–2007.
52. Montgomery RR, Gill JC, Scott JP. Hemophilia and von Willebrand disease. In: Nathan DG, Ginsburg D, Look AT et al., eds. *Nathan and Oski's Hematology of Infancy and Childhood*, 6th ed. Philadelphia: W. B. Saunders 2003.
53. Warrier AI, Lusher JM. DDAVP: a useful alternative to blood components in moderate hemophilia A and von Willebrand disease. *J Pediatr*. 1983;102:228–33.
54. Mannucci PM, Franchini M, Castaman G et al. Italian Association of Hemophilia Centers. Evidence-based recommendations on the treatment of von Willebrand disease in Italy. *Blood Transfus*. 2009;7(2):117–26.
55. Ewenstein BM. Use of ristocetin cofactor activity in the management of von Willebrand disease. *Haemophilia*. 2001;7(suppl 1);10–5.
56. Favaloro EJ, Bonar R, Kershaw G et al. Laboratory diagnosis of von Willebrand's disorder: quality and diagnostic improvements driven by peer review in a multilaboratory test process. *Haemophilia*. 2004;10(3):232–42.
57. Favaloro EJ, Thom J, Baker R et al. Assessment of current diagnostic practice and efficacy in testing for von Willebrand's disorder: results from the second Australasian multilaboratory survey. *Blood Coagul Fibrinolysis*. 2000;11(8):729–37.
58. Hayes TE, Brandt JT, Chandler WL et al. External peer review quality assurance testing in von Willebrand disease: the recent experience of the United States College of American Pathologists proficiency testing program. *Semin Thromb Hemost*. 2006;32(5):499–504.
59. Kitchen S, Jennings I, Woods TA et al. Laboratory tests for measurement of von Willebrand factor show poor agreement among different centers: results from the United Kingdom National External Quality Assessment Scheme for Blood Coagulation. *Semin Thromb Hemost*. 2006;32(5):492–98.
60. Zimmerman TS, Hoyer LW, Dickson L et al. Determination of the von Willebrand's disease antigen (factor VIII related antigen) in plasma by quantitative immunoelectrophoresis. *J. Lab Clin Med*. 1975;86:152–59.

61. Laurell CB. Quantitative estimation of proteins by electrophoresis on agar gel containing antibodies. *Anal Biochem*. 1966;14:45–52.
62. Cejka AJ. Enzyme immunoassay for factor VIII related antigen. *Clin Chem*. 1982;28:1356.
63. Peyvandi R, Palla R, Lotta LA et al. ADAMTS-13 assays I thrombotic thrombocytopenic purpura. *J Thromb Haemost*. 2010;8:631–40.
64. Pengo V, Tripodi A, Reber G et al. Update of the guidelines for lupus anticoagulant detection. *J Thromb Haemost*. 2009;7:1737–40.
65. Adcock DM, Marlar RA. Activated partial thromboplastins time reagent sensitivity to the presence of the lupus anticoagulant. *Arch Pathol Lab Med*. 1992;116:837.
66. McGlasson DL, Fritsma GA. A comparison of six dilute Russell viper venom time. *Semin Thromb Hemost*. 2013;39(3):315–19. Lupus Anticoagulant Screen/Confirm Assay Kits.
67. Rauch J, Tannenbaum M, Janoff AS. Distinguishing plasma lupus anticoagulants from antifactor antibodies using hexagonal (II) phase phospholipids. *Thromb Haemost*. 1989;62(3):892–96.
68. Charles LA, McGlasson DL, Hawksworth BA et al. Evaluation of a modified procedure for STACLOT-LA for the confirmation of lupus anticoagulants. *Blood Coagul Fibrinolysis*.1994;5:601–4.
69. Peerschke EIB, Castellone DD, Ledford-Kraemer M et al. Laboratory assessment of factor VIII inhibitor Titer. *Am J Clin Pathol*. 2009;131:552–58.
70. Kasper CK, Aledort LM, Aronson D et al. Proceedings: a more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh*. 1975;34:612
71. Verbruggen B, Novakova I, Wessels H et al. The modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost*. 1995;73(2):247–51.
72. Lusher JM, Hillman CRL. Effect of inhibitors on factors assays. In: Triplett DA, ed. *Advances in Coagulation Testing: Interpretation and Application*. Chicago: American Society of Clinical Pathology; 1986.
73. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. *Br J Haematol*. 2005;129:307–21.
74. Bovill EG. Systemic fibrinolysis. In: Goodnight S, Hathaway W, eds. *Disorders of Hemostasis & Thrombosis: A Clinical Guide*, 2nd ed. Columbus, OH: McGraw-Hill Professionals. 2000; 249–55.
75. Anand SS, Yi Q, Gerstein H et al. Relationship of metabolic syndrome and fibrinolytic dysfunction to cardiovascular disease. *Circulation*. 2003;108:420–25.
76. Eichinger S, Minar E, Bialonczyk C et al. D-dimer levels and risk of recurrent venous thromboembolism. *JAMA*. 2003;290:1071–74.
77. Sadanaga T, Sadanaga M, Ogawa S. Evidence that D-dimer levels predict subsequent thromboembolic and cardiovascular events in patients with atrial fibrillation during oral anticoagulant therapy. *J Am Coll Cardiol*. 2010;55:2225–231.
78. Waser G, Kathriner S, Wuillemin WA. Performance of the automated and rapid STA Liatest D-dimer on the STA-R analyzer. *Thromb Res*. 2005;116:165–70.
79. Reber G, Bounameaux H, Perrier A et al. Evaluation of advanced D-dimer assay for the exclusion of venous thromboembolism. *Thromb Res*. 2002;107:197–200.
80. Bauer, KA. Inherited disorders of thrombosis and fibrinolysis. In: Nathan DG, Ginsburg D, Look AT et al., eds. *Nathan and Oski's Hematology of Infancy and Childhood*, 6th ed. Philadelphia: W. B. Saunders; 2003.
81. Amiral J, Grosley B, Boyer-Neumann C et al. New direct assay of free protein S antigen using two distinct monoclonal antibodies specific for the free form. *Blood Coagul Fibrinolysis*. 1994;5(2):179–86.
82. Aiach M, Emmerich JE. Thrombophilia genetics. In: Coleman RW, Marder VJ, Clowes AW et al., eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2006.
83. Endler G, Kyrle PA, Eichinger S et al. Multiplexed mutagenically separated PCR: simultaneous single-tube detection of the factor V R506Q (G1691A), the prothrombin G20210A, and the methylenetetrahydrofolate reductase A223V (C677T) variants. *Clin Chem*. 2001;47:333–35.
84. International Committee for Standardization in Hematology, International Committee on Thrombosis and Haemostasis. ICSH/ICTH recommendations for reporting prothrombin time in oral anticoagulant control. *Thromb Haemost*. 1985;54:155.
85. McGlasson DL. Monitoring Coumadin—the original oral anticoagulant. *Clin Lab Sci*. 2013;26(1):43–47.
86. McGlasson DL. Using a single calibration curve with the anti-Xa chromogenic assay for monitoring heparin anticoagulation. *Lab Med*. 2005;36:1–3.
87. Olson JD, Arkin CF, Brandt JT MT et al. College of American pathologists Conference XXXI on Laboratory Monitoring of Anticoagulant Therapy: laboratory monitoring of unfractionated heparin therapy. *Arch Pathol Lab Med*. 1998;122:782–98.
88. Brill-Edwards P, Ginsberg JS, Johnston M et al. Establishing a therapeutic range for heparin therapy. *Ann Intern Med*. 1993;119:104–9.
89. Berry BB, Geary DL, Jaff MR. A model for collaboration in quality improvement projects: implementing a weight-based heparin dosing nomogram across integrated health care delivery system. *J Qual Improv*. 1998;24:459–69.
90. Rosborough TR, Shepherd ME. Achieving target antifactor Xa activity with a heparin protocol based on sex, age, height, and weight. *Pharmacotherapy*. 2004;24(6):713–19.
91. Ignjatovic V. Thrombin clotting time. *Methods Mol Biol*. 2013;992:131–38.
92. McGlasson DL, Kaczor DA, Krasuski RA et al. Effects of pre-analytical variables on the anti-activated factor X chromogenic assay when monitoring unfractionated heparin and low molecular weight heparin anticoagulation. *Blood Coagul Fibrinolysis*. 2005;16:173–76.
93. McGlasson DL, Chen MA, Fritsma GA et al. Urinary 11-dehydrothromboxane B2 levels in health individuals following a single dose response to two concentrations of aspirin. *J Clin Lig Assay*. 2005;28:147–50.
94. Nair SC, Dargaud Y, Chitlur M et al. Tests of global haemostasis and their applications in bleeding disorders. *Haemophilia*. 2010;16(Suppl 5): 85–92.
95. Hemker HC, Giesen P, AlDiere R et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb*. 2002;32:249–53.
96. McGlasson, D. Coagulation instrumentation. In Rodak BF, Fritsma GA, Keohane EM, eds. *Hematology: Clinical Principles and Applications*, 4th ed. St. Louis, MO: Elsevier, 2012.
97. Huzaidi AM. Evaluation and use of coagulometers. In: Kitchens S, ed. *Diagnosis of Hemophilia and Other Bleeding Disorders. A Laboratory Manual*, 2nd ed. Montreal, Quebec: The World Federation of Hemophilia; 2010;137–43. http://www.scribd.com/doc/75000922/401/Evaluation-and-use-of-coagulometers#page=142; accessed November 12, 2013.
98. Koepke, J. Technologies for coagulation instruments. *Lab Med*. 2000;31:211–16.
99. *Diagnostic Stago STA-R Evolution® Application Training Manual*. Parsippany NJ: Diagnostica-Stago; 2005.
100. Dabkowski B. From the new to the improved. *CAP Today*. 2012;26(1): 19–34. www.cap.org/apps/docs/cap_today/0112/0112_coagulation_analyzers.pdf; accessed November 18, 2013.100.



37 Hematology Procedures

CHERYL BURNS, MS
MARY ANN DOTSON, BS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Identify the three anticoagulants used in the hematology laboratory and give examples of laboratory tests that should be performed on blood anticoagulated with each.
2. Explain the mechanism of preventing coagulation for each anticoagulant.
3. Identify equipment and supplies required for phlebotomy.
4. Describe Occupational Safety and Health Administration (OSHA) standards related to phlebotomy.
5. List factors affecting the collection of a blood sample.
6. Determine the sequence of draw of phlebotomy collection tubes and correlate the collection technique of a blood sample with potential problems in sample analysis.
7. Describe the proper disposal of contaminated equipment and supplies.
8. Identify the component parts of a microscope and explain their functions.
9. Discuss preventive maintenance procedures for the microscopes.
10. Describe the peripheral blood smear preparation methods.
11. Identify the characteristics of an optimally prepared peripheral blood smear.
12. Describe the Romanowsky staining technique.
13. Recognize the characteristics of a properly stained peripheral blood smear.
14. Discuss the potential causes of improperly stained peripheral blood smears.
15. List the components included in a complete peripheral blood smear examination.
16. Correlate erythrocyte indices, leukocyte count, and platelet count with peripheral blood smear observations.
17. Determine the corrected leukocyte count based on the presence of nucleated erythrocytes.

(continued)

Chapter Outline

Objectives—Level I *(continued)*

18. State the principle of each test: cell enumeration by hemacytometer, hemoglobin concentration, hematocrit, erythrocyte sedimentation rate, reticulocyte count, solubility test for hemoglobin S, hemoglobin electrophoresis, acid elution for hemoglobin F, and osmotic fragility.
19. For each test listed in Objective 18, describe the procedure, identify potential sources of error, determine appropriateness of use including reflex testing, calculate and interpret results, and explain its clinical significance.
20. Calculate the erythrocyte indices.
21. Correlate the erythrocyte indices with complete blood count (CBC) data and peripheral blood smear examination.
22. Calculate the reticulocyte count.
23. Identify the reference intervals for each test in terms of gender and age.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Describe factors that influence brightfield microscopy.
2. Explain the principle of phase contrast microscopy.
3. Choose corrective actions for an improperly prepared or stained peripheral blood smear.
4. Detect abnormalities on peripheral blood smear examination, assess how they can alter cell count results (i.e., presence of nucleated erythrocytes or platelet satellitism), and recommend corrective action to ensure valid results.
5. State the principle of each of the following tests: quantitation of hemoglobin F and hemoglobin A_2 heat denaturation test for unstable hemoglobin, Heinz body stain, Donath-Landsteiner test for paroxysmal cold hemoglobinuria (PCH), erythropoietin assay, soluble transferrin receptor, and cytochemical stains (e.g., myeloperoxidase).
6. For each test listed in Objective 5, describe the procedure, identify potential sources of error, determine appropriateness of use including reflex testing, interpret results, and explain the clinical significance of the test.
7. For each test discussed in this chapter, correlate abnormal values with clinical conditions.
8. Select the cytochemical stain appropriate for confirming cell lineage and diagnosis in hematopoietic neoplasms.

Key Terms

Acute phase reactant
Anticoagulant
Anemia
Beer-Lambert's law
Biphasic antibody
Cold agglutinin disease
Column chromatography
Coverglass smear
Densitometer
Edematous
Hemoconcentration
Isoelectric focusing
Isopropanol precipitation
Leukocyte alkaline phosphatase (LAP)
Mean cell hemoglobin (MCH)
Mean cell hemoglobin concentration (MCHC)
Mean cell volume (MCV)
Multiple myeloma
Optimal counting area
Platelet clump
Platelet satellitism
Point-of-care testing (POCT)
Polycythemia
Reference interval
Refractive index
Romanowsky-type stain
Rouleaux
Rule of three
Smudge cell
Supernatant
Supravital stain
Tartrate-resistant acid phosphatase (TRAP)
Turnaround time (TAT)
Unique identification number
Wedge smear

Background Basics

The information in this chapter builds on concepts learned in other chapters. To maximize your learning experience, you should review and thoroughly understand these concepts before starting this unit of study:

Level I

- Describe normal leukopoiesis. (Chapter 7, 8)
- Describe normal erythropoiesis. (Chapter 5)
- Summarize the basics of hemoglobin synthesis and structure. (Chapter 6)
- Outline the classification systems for the anemias. (Chapter 11)

Level II

- Describe potential causes for changes in leukocyte concentrations. (Chapters 7, 8)
- Describe potential causes for decreased erythrocyte concentrations. (Chapter 5)
- Recognize the erythrocytic morphologic changes associated with size, shape, inclusions, and patterns of distribution. (Chapters 5, 10, 11)
- Recognize neutrophil and monocyte morphology and the reactive changes associated with lymphocytes. (Chapters 7, 8)
- Recognize and describe the morphologic characteristics of the developing erythrocytes and leukocytes. (Chapters 5, 7, 8)
- Recognize and describe normal bone marrow architecture. (Chapters 4, 38)
- Describe the hemoglobinopathies including hemoglobin S and hemoglobin C. (Chapter 13)
- Compare and contrast α thalassemia, β thalassemia, and hereditary persistence of fetal hemoglobin. (Chapter 14)
- Describe anemias characterized by membrane defects, including the pathophysiology of hereditary spherocytosis. (Chapter 17)
- Describe immune hemolytic anemias, including hemolytic disease of the newborn, transfusion-induced hemolytic anemia, cold hemagglutinin disease, and paroxysmal nocturnal hemoglobinuria. (Chapter 19)
- Describe the anemias of disordered iron metabolism and heme synthesis, including iron-deficiency anemia and anemia of chronic disease. (Chapter 12)
- Describe the etiology, pathophysiology, and classification of
  - Myeloproliferative disorders (Chapter 24)
  - Acute myelocytic leukemia (Chapter 26)
  - Acute lymphocytic leukemia (Chapter 27)
  - Lymphoid malignancies (Chapter 28)

OVERVIEW

Hematology tests are some of the most frequently ordered laboratory tests. The results obtained from these laboratory procedures are utilized in the diagnosis of a variety of disorders including anemias, leukemias, infections, and inherited leukocyte disorders. The laboratory professional must be able to perform these tests, verify results, solve problems related to erroneous results, correlate abnormalities with disease states, and suggest reflex tests when appropriate.

The two levels of testing in the hematology laboratory are routine and reflex. *Routine tests* such as the complete blood count (CBC) (Chapter 10), differential, and reticulocyte count are used as screening tests to determine the presence of a primary disease in the hematopoietic system or to identify hematologic changes that provide clues to the presence of nonhematologic diseases. Analysis of the results of these screening tests with the patient's clinical signs and symptoms help to determine whether further testing is necessary. Algorithms or critical pathways assist in the choice of follow-up testing (reflex testing) to abnormal screening test results. These reflex tests are used to definitively diagnose the patient's disease state. With a definitive diagnosis, the appropriate treatment plan can be implemented and monitored with additional laboratory procedures such as the reticulocyte count and immature reticulocyte fraction (IRF).

The chapter begins with a discussion of the regulations regarding laboratory testing. This is followed by a description of sample collection and care and use of the microscope. Next routine hematology tests are discussed, and concludes with a discussion of the laboratory procedures used in reflex testing. The principles, procedures, and results of the tests for specific clinical conditions are summarized. Test results are reported in conventional units with SI units given in parentheses. Detailed procedures for selected tests (formatted according to Clinical Laboratory Standards Institute guidelines) are provided in this chapter's Companion Resources.

LABORATORY TESTING REGULATIONS

Laboratory testing is performed either in a centralized laboratory (e.g., hospital or reference laboratory) or at a site close to the patient that is referred to as **point-of-care testing (POCT)**. It can be located in an intensive care unit (ICU), emergency department (ER), or a physician's office laboratory. POCT continues to increase as manufacturers develop smaller and more accurate instrumentation, increase the number of available tests (including waived and moderate complexity tests), and provide connectivity with laboratory information systems (LIS) and hospital information systems to improve the documentation and monitoring of results.[1] An advantage of POCT over centralized laboratory testing is that POCT can provide a critical test result to the physician for rapid assessment of the patient's clinical condition and initiation of appropriate therapeutic intervention. This can lead to better patient outcomes and lower health care costs by decreasing the length of the patient's hospital stay. The centralized laboratory is often unable to achieve rapid **turnaround time (TAT)** for certain critical tests.

No matter where the laboratory tests are performed, the facility must meet the regulations for quality laboratory testing outlined in the Clinical Laboratory Improvement Amendments 1988 (CLIA '88) and must have a certificate to perform laboratory testing. The type

of certificate and level of regulations vary depending on the tests that will be performed and its test category. The four test categories include waived tests, moderate complexity tests, high complexity tests, and provider-performed microscopy procedures. CLIA '88 defines a waived test as one that uses a simple and accurate methodology with low risk of erroneous result (e.g., hemoglobin determination by Hemocue®); has been approved by the Food and Drug Administration (FDA) for home use; has minimal quality control regulations; and has no requirement for on-site inspections. Laboratories performing only waived tests must apply for a certificate of waiver from their state agency (www.cms.hhs.gov/clia). There is an exception to the "no on-site inspection" clause. If waived tests are performed as part of a POCT program within a facility that is accredited by The Joint Commission or College of American Pathologists, waived tests are included in the inspection of the program and must meet criteria delineated in the accrediting agency's checklist for POCT.[1]

The FDA classifies moderate and high complexity tests (collectively known as *nonwaived* tests) based on the analyte to be tested, the specific methodology, and the expertise needed to perform the test method. For example, complete blood count (CBC) is classified as a moderate complexity test. Facilities performing nonwaived tests must meet strict regulations regarding quality assessment and quality control, personnel qualifications, proficiency testing, and competency testing (Chapter 43). These facilities must undergo an on-site inspection by a Centers for Medicare and Medicaid Services (CMS) authorized accrediting agency (e.g., College of American Pathologists) prior to receiving the certificate of accreditation and biannually thereafter to determine compliance with the CLIA '88 regulations. A certificate of compliance is issued to a facility if it is directly inspected by the CMS to determine compliance.

To be classified as a provider-performed microscopy procedure (PPMP), the laboratory test must be classified as of moderate complexity and must require the use of a microscope (e.g., microscopic urinalysis). Routine on-site inspections are not required, but these facilities are subject to nonwaived testing requirements and can be inspected as part of a routine inspection for nonwaived tests or if a complaint is alleged. Facilities that perform PPMP must apply for a certificate for PPMP from their state agency. This certificate allows a facility to perform PPMP and waived tests.

SAMPLE COLLECTION: PHLEBOTOMY

The phlebotomy procedure is important to all laboratory testing. The sample's quality dictates the accuracy of its final result. Laboratory professionals should have a thorough understanding of each sample collection technique. This section reviews the various aspects of phlebotomy or sample collection.

Anticoagulants

Most tests performed in the hematology laboratory involve anticoagulated blood. Once the blood has left the body, a series of reactions occur causing blood to clot within minutes. To prevent coagulation from occurring, a substance called an **anticoagulant** is mixed with the blood. Three anticoagulants are used in the hematology laboratory: (1) ethylenediaminetetraacetic acid (EDTA), (2) sodium citrate, (3) heparin.[2] Sample collection tubes are color coded by stopper to indicate the type of anticoagulant in the tube (Table 37-1 ★).

EDTA is the most commonly used anticoagulant in the routine hematology laboratory. EDTA prevents coagulation by chelating calcium, a necessary component of coagulation (Chapter 32). Its removal inhibits the coagulation process. The optimal concentration is 1.5 mg EDTA/mL blood. Sample collection tubes can contain one of three different salt forms of EDTA: disodium, dipotassium, or tripotassium. The sample collection tube's stopper is color coded in lavender to indicate the presence of EDTA.

Tests using EDTA samples include CBC, peripheral blood smear examination, reticulocyte count, and immunophenotyping of cells by flow cytometry. Ideally, the sample should be used within 6 hours of collection for the majority of these tests. The sample's stability can be extended to 24 hours with storage at 4°C for certain tests such as the CBC. If peripheral blood smears are to be prepared, they should be made within 3 hours of collection. After 3 hours at room temperature, degenerative changes can be observed by examining a Wright-stained blood smear. These changes include leukocytes with vacuoles, irregular cytoplasmic borders, and irregularly shaped nuclei. Also, platelets increase in size. Excess anticoagulant associated with an underfilled collection tube causes erythrocyte shrinkage. Concentrations of more than 2 mg EDTA/mL blood cause false decreases in

★ **TABLE 37-1** Sample Collection Tubes, Their Anticoagulant, and Typical Laboratory Use

Color Code	Anticoagulant	Typical Laboratory Procedure
Red	No anticoagulant	Antibody detection
Light blue	Sodium citrate	PT and APTT
Mottled red	No anticoagulant but contains clot activator and gel to facilitate separation of serum from cells	Cholesterol
Green	Heparin	Osmotic fragility, electrolytes
Purple or lavender	EDTA, K_3 (liquid), or K_2 (spray-coated)	CBC (K_2 EDTA can also be used for routine blood bank procedures; see below)
Pink	EDTA, K_2 (spray coated)	Blood bank procedures and whole blood hematology procedures (different colored stopper for easy distinction of blood bank versus. hematology samples)
Gray	Potassium oxalate and sodium fluoride	Glucose

PT = prothrombin time; APTT = activated partial thromboplastin time; CBC = complete blood count; EDTA = ethylenediaminetetraacetic acid

the microhematocrit (i.e., centrifuged hematocrit) and erythrocyte sedimentation rate (ESR).

Sodium citrate is the recommended anticoagulant for coagulation studies. The Clinical and Laboratory Standards Institute (CLSI) recommends the use of 3.2% sodium citrate.[3] The sample collection tube's stopper is color coded in light blue to indicate the presence of sodium citrate. Sodium citrate prevents coagulation by binding calcium in a soluble complex (Chapter 32). The appropriate ratio of anticoagulant:blood is 1:9. Discussion of sodium citrate and its use in coagulation studies can be found in Chapter 36.

Lithium heparin is the CLSI-recommended salt of heparin to be used for laboratory testing.[2] The sample collection tube's stopper is color coded in green to indicate the presence of heparin. Heparin's interaction with antithrombin prevents coagulation. The interaction leads to the inhibition of thrombin (Chapter 32). The recommended concentration for sample collection tubes is 15–30 units heparin/mL blood.

Lithium heparin is specifically recommended for the following laboratory tests: ammonia, carboxyhemoglobin, blood gases, zinc, and electrolytes (i.e., sodium, potassium, chloride, bicarbonate). In hematology, lithium heparin is the appropriate anticoagulant for the osmotic fragility test. The use of heparin for routine hematology procedures is not appropriate. Heparin can affect the platelets and leukocytes, causing them to clump. In addition, heparin causes morphologic distortion of platelets and leukocytes and tends to cause a bluish discoloration of the background of blood films stained with a Romanowsky stain such as Wright stain.

☑ CHECKPOINT 37-1

Contrast the mechanisms of anticoagulation for EDTA and heparin.

Equipment

The equipment needed for blood collection by venipuncture includes sample collection tubes, needles, tube holders or syringes, and a tourniquet. Lancets are used for capillary puncture. Other miscellaneous supplies are described in this section.

Sample Collection Tubes

Evacuated sample collection tubes are sterile and color coded to indicate the type of anticoagulant present or the lack of anticoagulant (Web Figure 37-1, Table 37-1). The Occupational Safety and Health Administration (OSHA) recommends the use of plastic sample collection tubes whenever possible. Collection tubes are manufactured in a variety of sizes to collect different volumes of sample (1 mL, 3 mL, 5 mL, etc.). The variety of volumes available minimizes the removal of excess amounts of blood. Today's hematology and chemistry instruments require only small amounts of sample for analysis. The interior of the sample collection tube is a vacuum. When a needle pierces the stopper, the required amount of blood is drawn from the vein into the tube by the vacuum.[4] The tube might not fill properly if the vein collapses or if the needle is displaced from the lumen of the vein.

For capillary punctures, microcollection tubes are available with the same anticoagulants and color coded in the same manner as the evacuated collection tubes, but they do not contain a vacuum. The microcollection tubes contain special features that facilitate the collection of capillary blood into the tubes, such as capillary pipet tips (see Web Figure 37-2 for examples of microcollection tubes). These adaptations help to decrease the time that it takes to collect the capillary sample and decrease errors related to sample collection (e.g., presence of microclots or hemolysis).

Needles

Sample collection needles are sterile hollow shafts of stainless steel with a beveled tip. The most common needles used for blood collection are 20- or 21-gauge. The gauge of a needle relates to the diameter of its bore. A small-gauge needle has a large diameter. For example, an 18-gauge needle is used to collect donor blood for transfusions because its larger bore diameter permits a more rapid collection of blood and decreases the chance of hemolysis. Special needles are required for sample collection using the evacuated collection tube system for which the needle must be double ended. The needle is screwed into a tube holder. The long end of the needle is inserted into the vein and the short end punctures the collection tube stopper (inside the tube holder) and releases the vacuum, allowing blood to flow into the tube.

Tube Holders

The evacuated sample collection tube system requires a tube holder or adapter with a shape similar to a syringe. The needle is threaded into its position in the holder, and sample collection tubes are rested in the holder, semiattached by the shorter needle end (Figure 37-1 ■). The tube holder also facilitates the insertion of the needle into the vein.

Syringe

The syringe was once the principal method of obtaining a blood sample, but it is now used only for difficult phlebotomies or the collection of an arterial blood sample. Sterile plastic syringes are available in a variety of sizes (e.g., 1, 5, or 10 mL).

■ **FIGURE 37-1** Assembled evacuated collection tube system. The double-ended needle is threaded into the tube holder and the purple top collection tube is secured in the holder by the shorter end of the needle that has partially pierced the stopper. Blood replaces the vacuum in the collection tube when the vein is entered and the stopper is completely pierced.

Tourniquet

Various tourniquets including latex-free rubber straps and blood pressure cuffs are available. Placement of the tourniquet on the patient's upper arm increases the resistance in venous blood flow, resulting in the distension of veins below the tourniquet. The phlebotomist is then able to visualize and/or palpate the veins to identify the "good" vein. The tourniquet should never be left in place longer than 1 minute prior to phlebotomy because doing so results in discomfort to the patient and **hemoconcentration** of the blood sample.

Lancets

Various sterile disposable lancets are available for performing capillary puncture. Their design has been improved to minimize the discomfort and maximize the quality of the sample. Disposable lancets provide a controlled incision depth. The selection of a lancet depends on the individual's age. For example, a lancet with an incision depth of 1.8 mm is typically used for neonatal collection to minimize trauma; a device with an incision depth of 2.4 mm is appropriate for older children and adults.

Other Equipment

Other equipment required to perform a phlebotomy includes gloves, safety glasses, alcohol pads, gauze, adhesive bandages, and biohazard sharps containers. Gloves and safety glasses have become important protective equipment for the phlebotomist to prevent exposure to blood-borne pathogens. Alcohol pads are commonly used to cleanse and prepare the phlebotomy site. Gauze with applied pressure is used to stop bleeding after the needle has been removed. Adhesive bandages are used to protect the venipuncture or capillary puncture site. The biohazard sharps container is used for properly disposing of lancets and needles to minimize potential puncture wounds.

Venipuncture

The venipuncture phlebotomy technique removes blood from a vein for laboratory testing using a sterile needle and an evacuated sample collection tube system.[4] Prior to performing the venipuncture, the phlebotomist must identify the patient. The 2008 National Patient Safety Goals implemented by The Joint Commission requires that two patient identifiers be used when collecting blood samples.[5] For example, proper identification can be achieved by comparing the patient's name and **unique identification number** from his or her wrist band to the information written on the requisition form.

Next the phlebotomist assembles the appropriate equipment for performing the venipuncture. The vein is selected by placing the tourniquet on the patient's upper arm and inspecting the forearm for a prominent vein (see Web Table 37-1 for considerations in selection of venipuncture site). The median cubital vein is typically used, but the cephalic vein is also appropriate. The vein is cleansed with an alcohol pad and allowed to air dry. The venipuncture is accomplished by inserting the needle into the vein at a 15° angle to the forearm (Web Figure 37-3). A slight release in resistance can be detected when the vein is entered. Blood is collected into the appropriate evacuated collection tubes by pushing the tube until the stopper is punctured with the short end of the needle (see Web Table 37-2 for the correct collection sequence of evacuated tubes). The vacuum is released as blood enters the collection tube. When blood has been collected in each tube, the tourniquet is released, the needle is withdrawn, and pressure with gauze is applied on the venipuncture site. The needle is immediately discarded into the biohazard sharps container. Collection tubes containing anticoagulant should be thoroughly but gently mixed to prevent clotting of the sample. Each collection tube should be properly labeled with patient's name, unique identification number, collection date and time, and phlebotomist's initials. Finally, the patient should be checked one last time to verify that bleeding has ceased and an adhesive bandage is applied to the venipuncture site.

Capillary Puncture

The capillary puncture technique allows a small but adequate amount of blood to be obtained for laboratory testing.[6] This is especially important in the pediatric patient (i.e., infant) who has a lower total blood volume than older children and adults. Venipuncture in this patient population can result in anemia. The phlebotomist should properly identify the patient before proceeding with the capillary puncture technique. Selection of the capillary puncture site depends on the individual's age. The side of the heel is used for newborns, and a location slightly to the side of center and perpendicular to the fingerprint of the last segment of the third or fourth finger is chosen for an older child or adult. The puncture site should be warm but not **edematous**. Accumulated interstitial fluid in the edematous area contaminates the blood sample. Warming the site prior to the capillary puncture increases blood flow, primarily arterial, through the site. With the site identified, appropriate equipment is assembled, and the site is cleansed with an alcohol pad and allowed to air dry. The capillary puncture is made, and the lancet is immediately discarded into a biohazard sharps container. The first drop of blood is wiped away because it likely contains excess interstitial fluid that could result in a diluted blood sample. Subsequent drops of blood are collected into the appropriate microcollection tubes. When sufficient blood has been collected, gauze is placed on the puncture site and slight pressure applied. The microcollection tubes containing anticoagulant should be properly mixed to ensure adequate anticoagulation to prevent clot formation. Each microcollection tube should be properly labeled with the patient's name, unique identification number, date and time of collection, and phlebotomist's initials. An adhesive bandage is applied to the puncture site.

Phlebotomy Safety

OSHA mandates safety guidelines for phlebotomy. The 1992 Occupational Exposure to Bloodborne Pathogens Standard and its 2001 revised directive address this issue thoroughly.[7–9] These standards promote safety through education, good work practices, and use of personal protective equipment (PPE) and medical devices that minimize exposure risk. Each laboratory should develop an exposure control plan to address these issues. Laboratory professionals should receive instruction as to the potential for exposure to, the methods of preventing exposure to, and the postexposure treatment of blood-borne pathogens such as hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV).

For the phlebotomist, the educational program should include practical experience using PPE and the collection systems available to minimize risk of needle sticks. At a minimum, the phlebotomist's PPE should include gloves and safety glasses to decrease risk of exposure through cuts

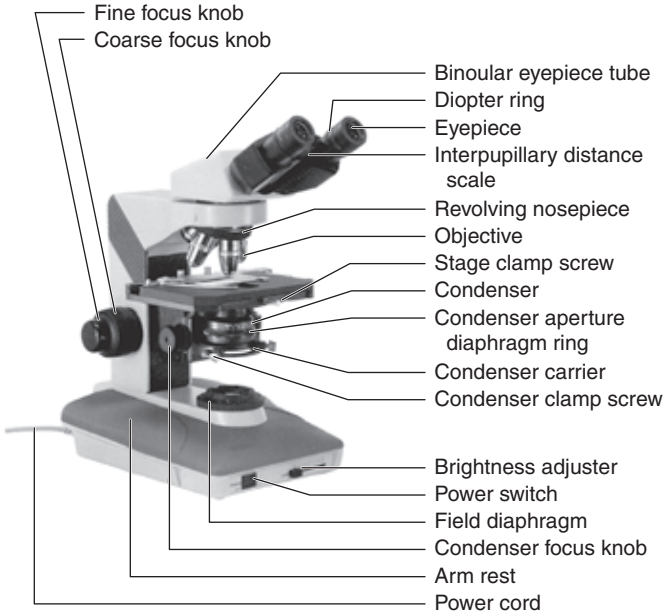
or abrasions on the skin and exposure through mucous membranes of the eyes. The revised directives require the evaluation and implementation of collection systems designed to reduce the risk of needle stick injury (Web Figure 37-4). Disposable lancet devices that retract the lancet into a protective guard after the capillary puncture has been made minimize the risk of a sharps injury from a lancet. Biohazard sharps containers should be readily available for disposal of lancets and needles.

An additional measure to reduce exposure to blood-borne pathogens is the use of plastic splashguards on the stoppers of sample collection tubes. The splashguards reduce the formation of aerosols that are created when the stopper is removed.

The entire exposure control plan for phlebotomy should be reviewed annually and changed as new devices become available to reduce the risk of sharps injury and as new treatments for blood-borne pathogens become available.

☑ CHECKPOINT 37-2

What are three steps a phlebotomist should take to minimize the risk of exposure to blood-borne pathogens when performing a venipuncture?



■ **FIGURE 37-2** Basic components of the compound microscope.

Image courtesy of Nikon Instruments Inc., Melville, NY.

MICROSCOPY: THE MICROSCOPE

The compound microscope is an essential instrument in the routine hematology laboratory. Its proper use and regular preventative maintenance are critical to the reliability of the results obtained from its use. Therefore, individuals utilizing the microscope need to be knowledgeable in its basic principles, operation, and preventative maintenance.

Bright-Field Microscopy

Bright-field microscopy is used extensively to examine stained blood and bone marrow samples. This section describes its components and aberrations that affect the quality of the sample examination.

Principles of the Compound Microscope

Any discussion of the principles of the compound microscope must begin with a review of its components, including eyepieces, binocular eyepiece tube, objectives located on the revolving nosepiece, microscope stage, condenser, condenser diaphragm, field diaphragm, and light source (Figure 37-2 ■). The condenser functions to direct the beam of light from the light source onto the sample. As the light rays illuminate the sample, they are altered and light is diffracted. The sample image is produced by a combination of the diffracted light and background light from the light source.[10–12]

The compound microscope's magnifying system uses two sets of lenses to form an enlarged image.[11,12] The first lens system is the objective, which projects a primary image plane to a location approximately 1 cm from the top of the microscope's body. The distance from the objective's back focal plane to the eyepiece is termed the *optical tube length* (160 mm). The second lens system is the eyepiece located above the primary image plane. The total magnification is the product of the magnification of the first and second lens systems (objective magnification times eyepiece magnification).

The resolving power (resolution) of a lens is its ability to distinguish two separate objects located close to one another and reveal the fine detail in a specimen.[11,12] It is a function of the numerical aperture (NA) of the lens and the wavelength (λ) of the illuminating light. The numerical aperture is a designation of the amount of light entering the objective from the microscope field. The NA of the substage condenser should be equal to or higher than the NA of an objective; otherwise, interference effects occur. Because the illuminating light remains constant in light microscopy, the numerical aperture determines the resolving power. That is to say, the higher an objective's NA, the greater is its resolving power.

☑ CHECKPOINT 37-3

What is the total magnification when a 10× objective is combined with a 10× eyepiece?

Lens Aberrations

An *aberration* is an optical defect that degrades the quality of an image.[11,12] Three important types of aberrations are associated with the objectives:

1. *Chromatic aberrations* give rise to color fringes and poor image definition. These aberrations result from the inability of the lens to bring the different wavelengths of light into focus at a single focal point. For example, blue light is brought to a focal point closer to the lens as compared with red light.
2. *Spherical aberrations* give rise to poor image definition and loss of contrast. In them, the light is refracted by the lens depending on the area (thickness) of the lens it passes through. For example, the light passing through the periphery of the lens is refracted more than light passing through the center. Therefore, the refracted light from the periphery is brought to a shorter focal point than the light passing through the center. This aberration becomes worse as the lens becomes thicker.

3. *Field curvature aberrations* result in the periphery of the field being slightly out of focus when the center is in focus. They are the result of the image in the focal plane being slightly curved by the objective.

To compensate for these aberrations, specialized lenses are used. The achromat lens compensates for chromatic aberrations at two colors and spherical aberrations at one color. The apochromat lens compensates for chromatic aberrations at three colors and spherical aberrations at two colors. The field curvature aberrations can be eliminated with the use of a flat-field (plan) objective lens. A plan apochromat lens is the highest grade, giving exceptional definition, superior color reproducibility, and prominent image flatness. This lens is most useful in the examination of morphologic detail.

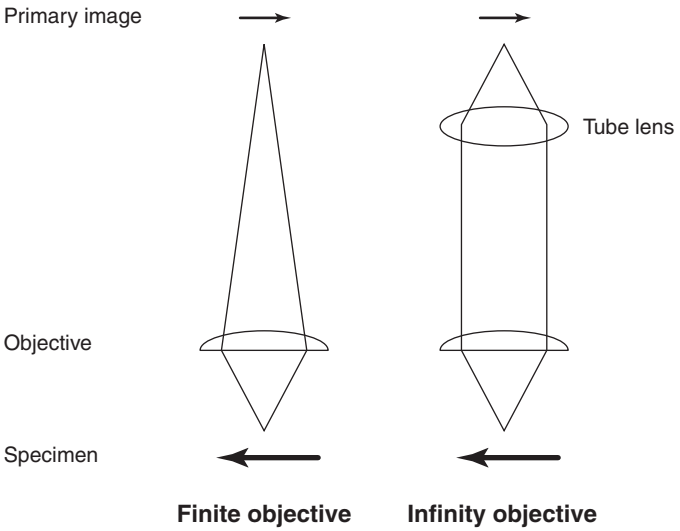
Infinity Optical System

Microscopes with the infinity optical system are available from most manufacturers. In this system, light that passes through the objective becomes a flux of parallel light rays.[13,14] These light rays do not converge to form the primary image until they pass through the tube lens (Figure 37-3 ■). In other words, the infinity objective sends the primary image to "infinity." For comparison, the finite objective projects light rays that converge within the tube length of the microscope to create the primary image (Figure 37-3). Two major advantages of the infinity optical system are the increased sharpness and clarity of the images and the increased flexibility of the microscope system. The increased sharpness and clarity result from higher numerical apertures and longer working distances (i.e., distance from the objective to the surface of the coverslip when the sample is sharply focused).

The infinity space associated with these systems allows for the addition of intermediate attachments without loss of optical performance such as an epi-fluorescence attachment for the evaluation of fluorescent stains.

Phase-Contrast Microscopy

With bright-field microscopy, unstained cells are difficult to examine because of the lack of absorption differences between cellular structures and undetectable variations in the **refractive index**. Examination of unstained cells can be accomplished using phase-contrast microscopy, which is based on the premise that transparent objects (e.g., unstained cells) cause a change in the phase of transmitted light (i.e., phase shift) because of the scattering and deflection of light.[12,14] When the transmitted light is shifted by one-quarter wavelength, these changes can be visualized as differences in light intensity. The two important components of a phase microscope are the annular diaphragm, or annular ring, located in the condenser and the phase-shifting element, or phase ring, located in the objective. The annular ring directs light through its open circular area, creating a hollow cone of light with a dark center that illuminates the sample (Figure 37-4 ■). The phase ring within the objective retards the wavelength of the deflected light by one-quarter and absorbs the nondiffracted light. This phase shifting occurs in the shaded area (Figure 37-4). As a result, light waves become out of phase. Maximum contrast between the cell and its surroundings is achieved when light waves are out of phase by one-quarter of a wavelength. With phase-contrast microscopy, cellular components that possess a higher refractive index than the surrounding environment appear dark, whereas components that possess a lower refractive index than the surrounding environment appear bright.



■ **FIGURE 37-3** Finite objective versus infinity objective. In the finite objective, light rays projected from the objective converge to form the primary image within the optical tube. For the infinity objective, light is projected as parallel rays from the objective. These rays converge to form the primary image only after they pass through the tube lens. The infinity space as defined by the distance between the objective and the tube lens can vary from 160 mm to 200 mm.

For proper operation, the annular ring must be centered to the phase ring.

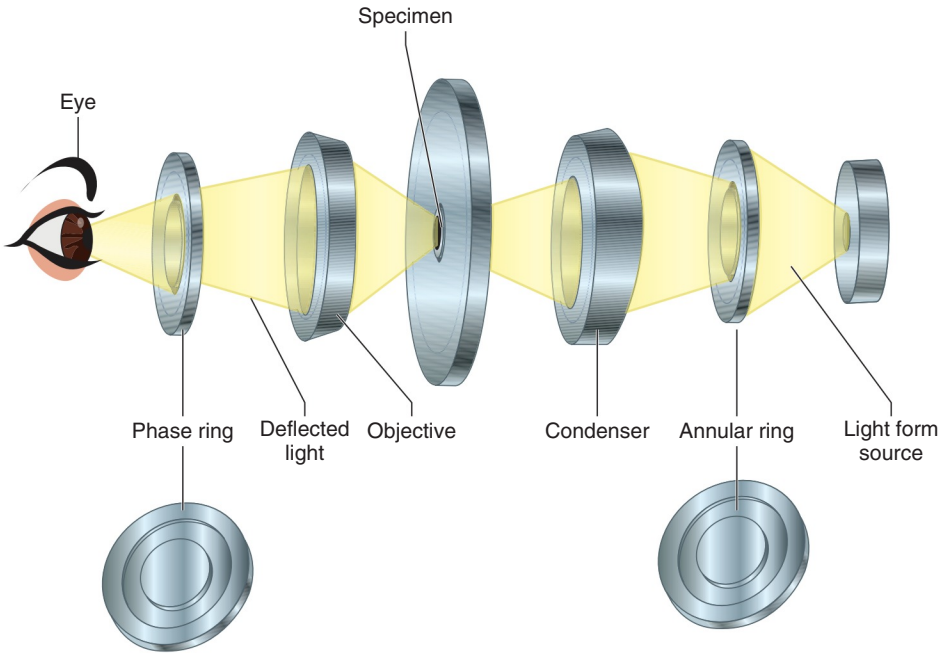
Koehler Illumination

Koehler illumination uses a double diaphragm illumination with the field diaphragm and the condenser diaphragm. The condenser diaphragm determines the resolution, contrast, and depth of field. Closing the condenser diaphragm increases the contrast and depth of field but decreases the resolution. The field diaphragm determines the illuminated area on the sample surface in relation to the microscope's field of view. Koehler illumination adjusts the two diaphragms to give uniform illumination of the field of view and optimum contrast and resolution of a sample by focusing and centering the light path.[10,11]

The procedure for Koehler illumination varies slightly depending on the microscope manufacturer (see Web Table 37-3). This procedure should be performed daily before using the microscope and should be repeated for each objective because each objective has a different light requirement (higher NA objectives require correspondingly higher NA settings on the condenser).

Preventative Maintenance

Each laboratory should establish a regular preventative maintenance program. The microscope manufacturer's instruction manual provides a preventative maintenance checklist. Any preventative maintenance program should include (1) cleaning the oil immersion lens daily with lens tissue and cleaner, (2) dusting optical surfaces (eyepieces, condenser, field lens, and filters) using bursts of air or a soft camel's hair



■ **FIGURE 37-4** Phase contrast light pathway. The annular ring within the condenser diaphragm creates a hollow cone of light that illuminates the sample. Transmitted light is shifted one-quarter wavelength by the phase-shifting element in the objective, creating maximum contrast between a cell and its surroundings.

brush, (3) cleaning external surfaces using a mild liquid soap—avoid the use of any organic solvents such as ether, alcohol, or xylene, and (4) periodically inspecting objectives for stubborn smudges or scratches.[11,12]

☑ CHECKPOINT 37-4

How does the examination of a sample by phase-contrast microscopy differ from that of bright-field microscopy?

PART I

ROUTINE HEMATOLOGY PROCEDURES

PERIPHERAL BLOOD SMEAR PREPARATION

The morphologic evaluation of hematopoietic cells by light microscopy requires the preparation of a well-stained blood smear.[15–18] The accuracy of the morphologic evaluation depends in part on the quality of the blood smear.

Manual Method

The two manual methods for preparing blood smears are the **coverglass smear** and the **wedge smear**. The coverglass smear method provides a smear with even distribution of the leukocytes. This method's disadvantages are the difficulty in mastering the technique, the fragility of the coverglass, and the difficulty in staining the coverglass. The wedge smear is the method most commonly used in routine laboratory practice, but it is subject to poor leukocyte distribution with monocytes and neutrophils being drawn out of the **optimal counting area** (or critical area) to the feather edge. Although the leukocyte distribution is poor, the technique is easily mastered and the smears are less fragile and can be stored for extended periods of time. An advantage of the leukocyte distribution in a wedge smear is that it allows for the identification of abnormal cells that tend to locate on the edges of the smear.

Either EDTA-anticoagulated venous blood or capillary blood is acceptable for the preparation of a blood smear (see Web Table 37-4 for this procedure). An optimal blood smear has the characteristics listed in Table 37-2 ★ (see Web Figure 37-5 for examples of properly and improperly prepared blood smears). A common problem associated with blood smears is the failure to dry the blood smear in a timely manner. This results in contraction artifacts of the cells, especially with increased humidity. In certain physiological conditions including **anemia**, **polycythemia**, **multiple myeloma**, and **cold agglutinin disease**, making good blood smears is difficult because of the blood's

★ **TABLE 37-2** Optimal Blood Smear Characteristics

Minimum length 2.5 cm
Gradual transition in thickness from thick to thin
Straight feather edge
Margins narrower than slide
No streaks, waves, or troughs

★ **TABLE 37-3** Resolution of Problems in Preparation of a Blood Smear

Problem	Resolution
Presence of crenated erythrocytes	Dry smear quickly and thoroughly
Thin smear resulting from anemia	Increase spreader slide angle and increase push speed
Thick smear resulting from polycythemia	Decrease spreader slide angle and decrease push speed
Presence of agglutinated erythrocytes associated with cold agglutinin disease	Warm blood @ 37°C for 15 minutes before preparing smear
Increased viscosity associated with multiple myeloma	Decrease spreader slide angle and decrease push speed

abnormal composition (Table 37-3 ★). The thickness or thinness of the blood smear can be regulated by:

- Adjusting the amount of blood used to make the drop
- Altering the speed with which the drop is smeared
- Altering the angle at which the spreader slide is used

Automated Method

Automated methods for preparing blood smears have been developed for nearly all major hematology instruments on the market (Chapter 39). These methods are based on the wedge smear technique. Automated methods that are components of a hematology instrument combine blood smear preparation and staining. Advantages of these methods include minimal exposure to biohazardous material because many are closed-tube systems, increased consistency between blood smears, and increased optimal counting area.

A portable semiautomated instrument that is not specific for any given hematology instrument is also available. This instrument controls the speed and angle of the spreader blade, but the laboratory professional controls the size of the blood drop. Overall advantages and disadvantages of the automated methods are similar to those of the manual wedge smear method.

☑ CHECKPOINT 37-5

A laboratory professional consistently prepares thin blood smears resulting in minimal counting area. What would you suggest this individual do to improve the quality of these blood smears?

PERIPHERAL BLOOD SMEAR STAINING

For more than 100 years, **Romanowsky-type stains** have been used in the morphologic classification of hematopoietic cells.[19,20] A Romanowsky-type stained blood smear is extremely important in the hematology laboratory because a wealth of information can be obtained from the evaluation of a well-stained peripheral blood smear.

The combined action of methylene blue and its oxidation products and eosin Y or eosin B produces the Romanowsky staining effect. This yields a purple color to the nuclei of leukocytes and neutrophilic granules and reddish orange color to the erythrocytes. The principal components responsible for this effect are azure B (a methylene blue oxidation product) and eosin Y. The wide variation in purple and red shades seen with Romanowsky staining allows for subtle distinctions in cellular characteristics.

The staining properties of Romanowsky stains depend on the binding of dyes to chemical structures and the interactions between azure B and eosin Y. Acidic groupings of nucleic acids, the proteins of the cell nuclei, and immature or reactive cytoplasm binds azure B, the purplish basic dye. Eosin Y, the reddish acidic dye, binds to the basic groupings of the hemoglobin molecules and the basic proteins within certain granules.

☑ CHECKPOINT 37-6

Which component of Wright stain is responsible for staining hemoglobin within erythrocytes?

Examples of Romanowsky stains include Wright, Wright-Giemsa, Leishman, May-Grunwald, and Jenner stains. The most commonly used are Wright and Wright-Giemsa.

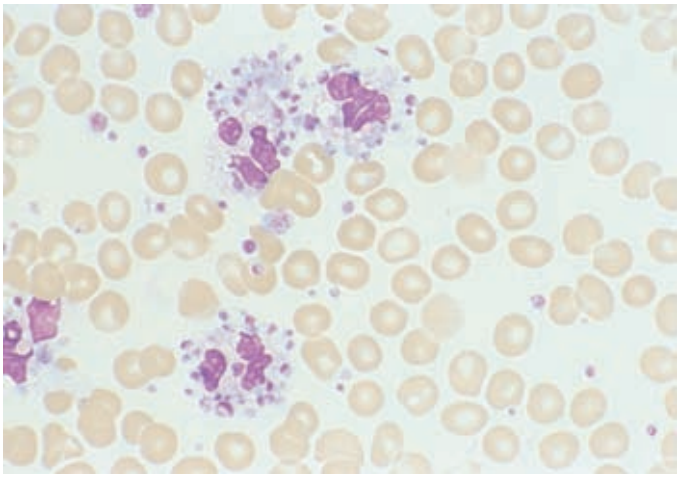
The staining process begins with the methanol fixation step, which results in the adherence of cellular proteins to the glass microscope slide, preventing the cells from being washed away during subsequent steps (see Web Table 37-5 for this procedure). Additional fixation takes place when the blood smear is flooded with Wright stain. The actual staining begins with the addition of buffer to the Wright stain, resulting in the ionization of the dyes. A properly stained blood smear meets the criteria outlined in Table 37-4 ★. Potential causes of an improperly stained blood smear are given in Table 37-5 ★.

★ **TABLE 37-4** Characteristics of a Properly Stained Blood Smear by Macroscopic and Microscopic Evaluation

Type of Evaluation	Characteristics
Macroscopic	Smear is pinkish purple in color.
Microscopic	Blood cells are evenly distributed.
	Areas between cells are clear.
	Erythrocytes are orange-red.
	Neutrophilic granules are pale purple.
	Eosinophilic granules are red-orange.
	Basophilic granules are purplish black.
	Lymphocytes' cytoplasm is blue.
	Leukocytes' nuclei are purple.
	Chromatin and parachromatin are distinct within the nucleus.
	Precipitated stain is minimal or absent.

★ **TABLE 37-5** Potential Causes of Improperly Stained Blood Smear

Problem	Potential Causes
Excessively blue or dark stain	Prolonged staining
	Inadequate washing
	Use of a stain and/or buffer with an alkalinity that is too high
	Thick blood smear
Excessively pink or light stain	Insufficient staining
	Prolonged washing
	Use of a stain and/or buffer with an acidity that is too low
Presence of precipitate	Unclean slides
	Drying during staining process
	Inadequate filtration of stain



■ **FIGURE 37-5** Platelet satellitism (peripheral blood; Wright-Giemsa stain; 1000× original magnification).

☑ CHECKPOINT 37-7

If the erythrocytes appear bluish gray and the leukocyte nuclei are black, how would you correct this problem?

PERIPHERAL BLOOD SMEAR EXAMINATION

The examination of a well-stained peripheral blood smear is one of the most frequently performed tests in the hematology laboratory.[15,21–23] The thorough examination of a peripheral blood smear can be used (1) as a screening tool to identify illness, (2) for making the definitive diagnosis of certain hematologic and nonhematologic conditions, and (3) to monitor the patient's response to therapy. The peripheral blood smear evaluation includes an estimation of leukocyte and platelet count, the detection of abnormal cells and abnormal erythrocyte distribution, the review of erythrocyte and platelet morphology, and a 100-cell leukocyte differential (Chapter 10; see Table 10-5 for a summary of this procedure). The detailed procedure is also provided in this chapter's Companion Resources.

On 100× magnification (10× objective), the peripheral blood smear is scanned to ensure even distribution of leukocytes and observe immature or abnormal cells, **smudge cells**, **platelet clumps**, **platelet satellitism**, and abnormal erythrocyte distribution patterns such as **rouleaux** or agglutination (Figure 37-5 ■, Table 37-6 ★). The leukocyte estimate is obtained by counting the number of leukocytes in each of five fields of view and applying the calculation formula (Figure 37-6 ■). The leukocyte estimate should correlate with the leukocyte count ±25%.

On 1000× magnification (100× objective), platelet morphology is observed, and a platelet estimate is obtained by counting the number of platelets in each of five fields of view and applying a calculation formula similar to that used for the leukocyte estimate (Figure 37-7 ■). The platelet estimate should correlate with the platelet count ±25%. Erythrocyte morphology is evaluated by carefully examining erythrocyte size, shape, color, and for the presence of inclusions. Normal erythrocytes are described as normocytic and normochromic, and this appearance correlates with normal erythrocyte indices. Any change beyond normal variation

★ **TABLE 37-6** Abnormalities Detected on Peripheral Blood Smear, Their Effect on Cell Counts, and Corrective Procedures to Take

Abnormality	Effect on Cell Counts	Corrective Action
Smudge cells	No effect on cell counts	Add 1 drop of 22% bovine albumin to 5 drops of blood, mix, and prepare blood smear.
Nucleated erythrocytes	Falsely elevated leukocyte count	Correct leukocyte count for presence of nucleated erythrocytes.
Platelet clumps	Falsely decreased platelet count	If collected in EDTA, recollect using citrate tube. Platelet count should be multiplied by 1.1 to correct for dilutional effect of liquid citrate.
Platelet satellitism	Falsely decreased platelet count	Use correction for platelet clumps.
Erythrocyte agglutination	Falsely decreased erythrocyte count	Warm blood at 37°C for 15 minutes and retest.
Rouleaux	No effect on cell counts	No correction is available

$$\frac{\text{Total number of leukocytes counted}^{a}}{5} \times 0.2 \times 10^3/\text{mcL}^{b}$$

$$= \text{Leukocytes} \times 10^3/\text{mcL}$$

Example: If total number of leukocytes counted = 150,

$$\frac{150}{5} \times 0.2 \times 10^3/\text{mcL} = 6.0 \times 10^3/\text{mcL}$$

■ **FIGURE 37-6** Calculation of leukocyte estimate. This formula is based on performing the estimate at 100× magnification. The estimation factor varies depending on the total magnification used as defined by the ocular and objective magnifications. Thus, each laboratory should determine or validate this factor based on its microscope.

[a]Total number of leukocytes counted in five fields at 100× (10× objective) magnification

[b]1 leukocyte = 0.2×10^3/mcL

should be noted because certain variations in the erythrocytes are characteristic of specific hematologic disorders. A detailed discussion of possible morphologic changes can be found in Chapters 10 and 11.

The leukocyte differential is performed by counting 100 cells per slide using the battlement track method for examination (see Figure 37-8 ■). Each leukocyte (Chapters 7, 8) encountered must be identified and placed in the appropriate category; distorted cells are included only if they are clearly identifiable. Nucleated erythrocytes (Chapter 5) are not included within the differential but are tabulated separately. The results of the differential are reported in the percentage of each type of leukocyte counted. The nucleated erythrocytes are expressed as the number per 100 leukocytes. If 5 or more nucleated erythrocytes are observed, the leukocyte count must be corrected for their presence because they are included in the automated leukocyte count (Figure 37-9 ■). Several automated hematology instruments can automatically correct leukocyte count for the presence of nucleated erythrocytes because these instruments are capable of recognizing and enumerating nucleated erythrocytes within a blood sample (Chapter 39). Finally, leukocytes are observed

$$\frac{\text{Total number of platelets counted}^{a}}{5} \times 15 \times 10^3/\text{mcL}^{b} = \text{Platelets} \times 10^3/\text{mcL}$$

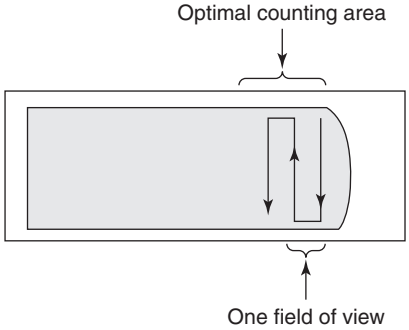
Example: If total number of platelets counted = 100,

$$\frac{100}{5} \times 15 \times 10^3/\text{mcL} = 300 \times 10^3/\text{mcL}$$

■ **FIGURE 37-7** Calculation of platelet estimate. This formula is based on performing the estimate at 1000× magnification. Refer to Figure 37-6 for a discussion of estimation factor.

[a]Total number of platelets counted in five fields at 1000× (100× objective) magnification

[b]If EDTA-anticoagulated blood, 1 platelet = 15×10^3/mcL; if capillary blood, 1 platelet = 20×10^3/mcL



■ **FIGURE 37-8** Pathway for the leukocyte differential. The differential is performed within the optimal counting area where the erythrocytes can be touching but not overlapping. As you approach the opposite side of the smear, the objective is moved into the body of the smear by one field of view. This pattern is followed until 100 leukocytes have been identified.

for changes in morphology (e.g., Döhle bodies, hypersegmented neutrophils; Table 10-7 ★, Chapters 21 and 22). These changes can be the result of an underlying hematologic disorder, the presence of excess anticoagulant associated with an underfilled collection tube, or the failure to prepare the blood smears within 3 hours of collection. Typical anticoagulant changes include cytoplasmic vacuolization, degranulation, karyorrhexis, karyolysis, and changes in nuclear shape.

The leukocyte differential reference intervals for children as well as adult males and females can be found in Table B inside the front cover.

☑ CHECKPOINT 37-8

In a platelet estimate, 76 platelets were observed in five fields on a stained blood smear. Calculate the platelet estimate. If the platelet count was 189×10^3/mcL (μL) (189×10^9/L), would these results correlate? Assume that the sample was collected with EDTA.

$$\frac{\text{Leukocyte count} \times 100}{100 + \text{Number of nucleated erythrocytes}^{a}} = \text{Corrected leukocyte count}$$

Example: Leukocyte count = 20.0×10^3/mcL
Nucleated erythrocytes/100 leukocytes = 10

$$\frac{20.0 \times 10^3/\text{mcL} \times 100}{100 + 10} = 18.2 \times 10^3/\text{mcL}$$

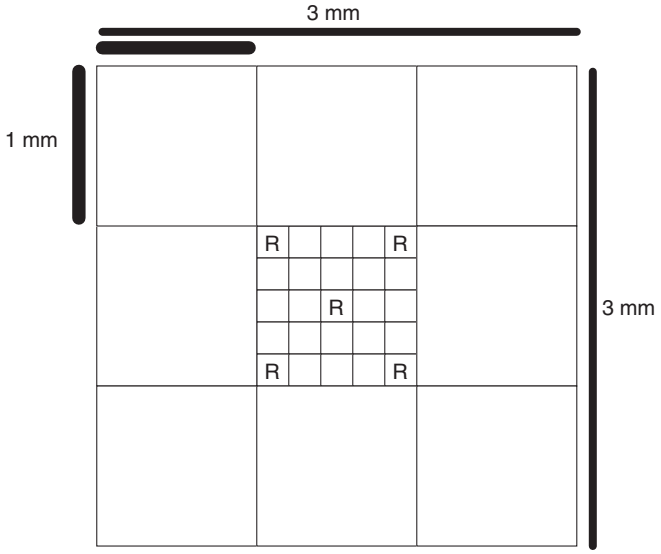
■ **FIGURE 37-9** Leukocyte count correction for presence of nucleated erythrocytes.

[a]Number of nucleated erythrocytes counted per 100 leukocytes at 1000× (100× objective) magnification

CELL ENUMERATION BY HEMACYTOMETER

Cell counts are performed manually by diluting blood with a diluent, loading a small amount of the diluted sample on a ruled device (hemacytometer), and counting the cells microscopically. The hemacytometer consists of two side-by-side identically ruled glass platforms mounted in a glass holder. Each platform contains a ruled square measuring 3 × 3 mm (9 mm^2) and is subdivided according to the improved Neubauer ruling (Figure 37-10 ■). This ruling subdivides the ruled square into nine large squares, each measuring 1 × 1 mm (1 mm^2). The four corner squares are used for leukocyte counts, and the large center square (1 mm^2) is used for platelet and erythrocyte counts. This center square is divided into 25 smaller squares, each with an area of 0.04 mm^2. The five small squares labeled *R* are used in performing the erythrocyte count, whereas the entire large center square is used in performing the platelet count.

On either side of the two ruled glass platforms is a raised ridge. The coverglass is placed on top of the ridge. The distance between the coverglass and the surface of the ruled area (depth) is exactly 0.1 mm. Thus, the ruled area on each side of the hemacytometer holds a volume of 0.9 mm^3 (3 × 3 × 0.1).



■ **FIGURE 37-10** Neubauer hemacytometer counting area. The entire counting area is 9 mm^2 (3 mm × 3 mm) and is divided into nine squares. Each square is 1 mm^2 (1 mm × 1 mm) in area. Using the 10× objective, 1 square of the counting area (1 mm^2) can be viewed. For the manual leukocyte count, the four corner squares are counted. The center square is further divided, and the *R* squares are used for erythrocyte counts, whereas the entire center square is used for platelet counts.

Manual Leukocyte Count

Whole blood is diluted with an acetate buffer (pH 3) solution containing gentian violet (or 3% acetic acid), which hemolyzes mature erythrocytes and stains the leukocyte's nucleus a light violet-blue color to facilitate leukocyte (white blood cell [WBC]) counting (www.bioanalytic.de).[24] The standard dilution for leukocyte counts is 1/20. The detailed procedure for the manual leukocyte count is provided in this chapter's Companion Resources. With proper light adjustment, the nuclei of the leukocytes should appear light violet-blue in color. The number of leukocytes in the four corner squares are counted using the 10× objective. Potential sources of error in performing hemacytometer cell counts are provided in Web Table 37-6.

The number of leukocytes is calculated per microliter ($\times 10^9$/L) of blood. To make this determination, the total number of cells counted must be corrected for the initial dilution of blood and the volume of blood used (Figure 37-11 ■). Because the dilution of blood for a leukocyte count is 1/20, the reciprocal of the dilution is 20 (i.e., dilution factor). The volume of blood used is based on the number of squares counted, the area of each square, and the depth of the solution. For the leukocyte count, the four corner squares are counted; each square's area is 1 mm^2, and the depth of the solution is 0.1 mm; therefore, the volume is 0.4 mm^3. Thus, the number of leukocytes counted in the four corner squares is multiplied by 50 (20/0.4) and reported as number of leukocytes per microliter ($\times 10^9$/L).

Reference intervals for leukocyte counts in adults and children can be found in Table B on the inside cover. Conditions commonly associated with increased or decreased leukocyte counts are shown in Table 37-7 ★ (Chapters 7, 8, 21, 22, and 24–28).

$$\frac{\text{Total nunber of cells counted} \times \text{Reciprocal of the dilution}}{\text{Number of squares counted} \times \text{Area of each square} \times \text{Depth of the solution}} = \text{Cells/mm}^3$$

Example: Total number of cells (one side of hemacytometer) = 200 cells
Dilution = 1/20
Number of squares counted = 4
Area of each square = 1 mm^2
Depth of solution = 0.1 mm

$$\frac{200 \times 20}{4 \times 1\ \text{mm}^2 \times 0.1\ \text{mm}} = 10{,}000/\text{mm}^3 \text{ mcL } (\mu\text{L}) \text{ or } 10.0 \times 10^9/\text{L}$$

■ **FIGURE 37-11** Calculation formula for hemacytometer cell counts.

★ **TABLE 37-7** Conditions Commonly Associated with Changes in Erythrocyte, Leukocyte, and Platelet Concentrations

	Increased Concentration	Decreased Concentration
Erythrocyte	Polycythemia	Anemia
	Myeloproliferative neoplasms	Acute leukemia
	Dehydration	Myelodysplastic syndromes
		Hemorrhage
		Hemolysis
Leukocyte	Bacterial infections	Viral infections
	Inflammation	Aplastic anemia
	Metabolic intoxication	Megaloblastic anemia
	Hemolysis	Drug-induced leukopenia
	Hemorrhage	Myelodysplastic syndromes
	Tissue necrosis	
	Strenuous exercise	
	Anxiety or stress	
	Acute leukemia	
	Myeloproliferative neoplasms	
Platelet	Splenectomy	Immune thrombocytopenia
	Hemorrhage	
	Iron deficiency anemia	Aplastic anemia
	Myeloproliferative neoplasms	Megaloblastic anemia
		Myelodysplastic syndromes
		Acute leukemia

Manual Erythrocyte Count

Manual erythrocyte counts are occasionally performed on blood and certain body fluids (Chapter 30) and are therefore included in this chapter. Whole blood is diluted with improved Hayem's reagent or isotonic saline, which prevents erythrocyte (red blood cell) lysis and facilitates erythrocyte counting (www.bioanalytic.de).[25] The standard dilution for erythrocyte counts is 1/200. For the manual erythrocyte count, erythrocytes are counted in five of the smaller squares within the large center square (*R*; Figure 37-10) using the high dry objective (40×). Potential sources of error are listed in Web Table 37-6.

The number of erythrocytes is calculated per microliter ($\times 10^9$/L) of blood using the calculation formula for hemacytometer cell counts (Figure 37-11). The variations are in the reciprocal of the dilution (i.e., dilution factor) and the volume. For erythrocyte counts, the reciprocal of the dilution is 200, and the volume is 0.04 mm^3 (10 squares counted, each square's area = 0.04 mm^2; depth = 0.1 mm) when the diluted sample from a single reagent vial is placed on both ruled areas of the hemacytometer.

The reference intervals for erythrocyte counts in adult males and adult females and children can be found in Table A on the inside cover. The reference intervals vary with age as shown. Conditions commonly associated with increased or decreased erythrocyte counts are shown in Table 37-7 (Chapters 5, 12–20, and 23).

Manual Platelet Count

Whole blood is diluted with a 1% oxalate buffer solution.[26] The diluent lyses all erythrocytes and disaggregates the platelets. The standard dilution for platelet counts is 1/100. The detailed procedure is provided in this chapter's Companion Resources. Platelets are counted in the large center square (1 mm^2) using high dry objective (40×). With phase contrast microscopy, the platelets appear as dark round or oval bodies because of their higher refractive index. Potential sources of error for manual platelet counts are given in Web Table 37-7.

The number of platelets is calculated per microliter ($\times 10^9$/L) of blood using the calculation formula for hemacytometer cell counts (Figure 37-11). The variations are in the reciprocal of the dilution factor and the volume. For platelet counts, the reciprocal of the dilution (i.e., dilution factor) is 100 and the volume is 0.2 mm^3 (two squares counted; each square's area = 1 mm^2; depth = 0.1 mm) when the diluted sample from a single reagent vial is placed on both ruled areas of the hemacytometer.

The reference interval for platelet counts is 150–400 × 10^3/mcL (150–400 × 10^9/L). Conditions commonly associated with increased or decreased platelet counts are shown in Table 37-7 and (Chapters 31, 16, 19, 25–27, and 33).

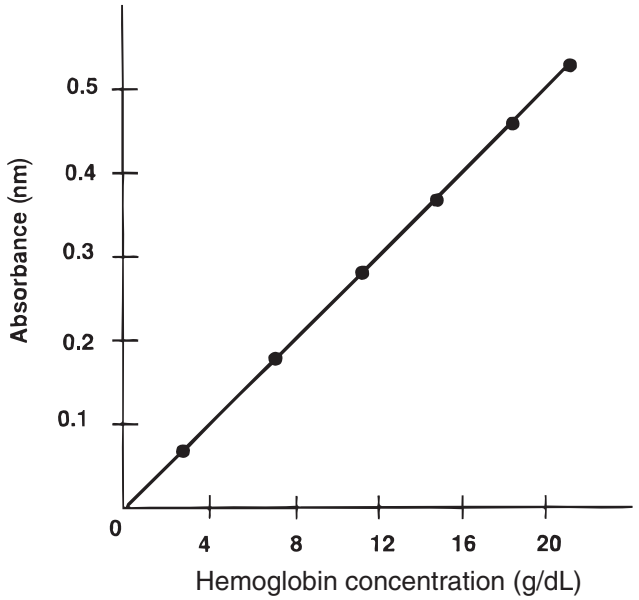
☑ CHECKPOINT 37-9

a. A manual platelet count was performed on an EDTA-anticoagulated blood sample using the standard dilution. The number of platelets counted from the first diluted vial was 125 and from the second diluted vial was 131. What is this patient's platelet count?

b. What are two possible physiologic causes or mechanisms that could lead to a decrease in this patient's platelet count?

HEMOGLOBIN CONCENTRATION

This procedure dilutes whole blood in cyanmethemoglobin (Drabkin's) reagent. The diluting fluid hemolyzes the erythrocytes, releasing hemoglobin into the solution. The ferrous ions (Fe^{++}) of the hemoglobin molecules are oxidized by potassium ferricyanide to ferric ions (Fe^{+++}). This oxidation results in the formation of methemoglobin, which combines with the cyanide ions (CN^-) to form cyanmethemoglobin, a stable compound that can be quantitated using spectrophotometry.[27,28] All hemoglobin derivatives except sulfhemoglobin are converted to cyanmethemoglobin. The detailed procedure is provided in this chapter's Companion Resources.



■ **FIGURE 37-12** Hemoglobin standard calibration curve. When using this standard curve, if the absorbance for a patient's hemoglobin determination is 0.250, the hemoglobin result is 10.0 g/dL.

When measured spectrophotometrically at 540 nm, the absorbance of cyanmethemoglobin follows **Beer-Lambert's law** and is directly proportional to the concentration of hemoglobin in the blood. A reference (standard) curve is prepared using cyanmethemoglobin standard solutions of known hemoglobin concentrations (Figure 37-12 ■). The hemoglobin concentration (patient or control) is read from the reference (standard) curve. The reference intervals for hemoglobin vary with age and sex. Conditions associated with changes in hemoglobin concentration are broadly divided into those associated with decreased levels, the anemias (Chapters 5, 11–20), and those associated with increased levels, the polycythemias (Chapters 5 and 24).

Several physiologic conditions lead to turbidity in the cyanmethemoglobin reagent–patient sample mixture (Table 37-8 ★). Any turbidity in the mixture results in falsely elevated values. It is important to recognize the falsely elevated result and take the appropriate corrective action to obtain the true hemoglobin value (Chapter 43). A falsely elevated hemoglobin result can be indicated by poor correlation between the patient's hemoglobin and hematocrit result (i.e., hemoglobin × 3 = hematocrit), as is discussed in the next section.

HEMATOCRIT

The hematocrit of a blood sample is the packed cell volume (PCV) denoting the percentage of erythrocytes in a known volume of whole blood.[29,30] The hematocrit is one of the simplest and most reproducible laboratory tests and is useful in detecting anemia and polycythemia.

In the microhematocrit method, a capillary tube is filled with anticoagulated whole blood and centrifuged in a microhematocrit centrifuge at 10,000–15,000 g for 5 minutes (see this chapter's Companion Resources for this detailed procedure). The volume occupied by the erythrocytes is expressed as a percentage of the total volume (PCV). For potential sources of error for this procedure, refer to Web Table 37-8. The reference intervals vary with age and sex as shown in Table A on the inside cover.

To ensure the accuracy of the hemoglobin and hematocrit, a quick mathematical check of hemoglobin × 3 = hematocrit is done. This **rule of three** is useful in the detection of measurement errors or patient sample issues such as the presence of lipemia. The rule of three is discussed in Chapter 10, and the potential causes of a mismatch between a patient's hemoglobin and hematocrit are described in Chapter 43 (Table 43-8 and Table 43-9).

ERYTHROCYTE INDICES

Wintrobe introduced the erythrocyte indices, **mean cell volume (MCV)**, **mean cell hemoglobin (MCH)**, and **mean cell hemoglobin concentration (MCHC)** in 1929. These indices are calculated from the erythrocyte count, hemoglobin concentration, and hematocrit (Table 37-9 ★). Reference intervals for the erythrocyte indices vary with age as shown in Table A on the inside cover. With the advent of automation in hematology, the erythrocyte indices are measured and/or calculated from data collected by erythrocyte analysis (Chapter 39). The electrical impedance counters measure the MCV by averaging the heights of the voltage pulses. The MCH, MCHC, and hematocrit are calculated from the measured values, MCV, hemoglobin, and erythrocyte count. The automated MCV eliminates the problem of a falsely elevated MCV as the result of trapped plasma between

★ **TABLE 37-8** Physiologic Conditions Leading to Error in Hemoglobin Determination and Corrective Actions to Take

Physiologic Condition	Corrective Action
Extremely high leukocyte count (>50.0 × 10^3/mcL)	Centrifuge hemoglobin mixture and use supernatant to determine hemoglobin concentration.
Presence of hemoglobin S or hemoglobin C	Use a 1/2 dilution of the hemoglobin mixture with distilled water to determine hemoglobin concentration (multiply hemoglobin by 2).
Presence of lipemia	Use a patient plasma blank or replace patient's plasma with isotonic saline to determine hemoglobin concentration.
Presence of abnormal globulins (e.g., those found in multiple myeloma or Waldenstrom's macroglobulinemia)	Increase the alkalinity of the cyanmethemoglobin reagent by adding potassium carbonate, and then repeat hemoglobin determination.

★ **TABLE 37-9** Erythrocyte Indices[a]

Calculation	Example	Reference Interval (conventional)
$MCV^{b} = \frac{\text{Hct (\%)}}{\text{RBC } (\times 10^6/\text{mcL})} \times 10$	$\frac{42.6}{4.88} \times 10 = 87$ fL	80–100 fL
$MCH^{c} = \frac{\text{Hb (g/dL)}}{\text{RBC } (\times 10^6/\text{mcL})} \times 10$	$\frac{14.2}{4.88} \times 10 = 29$ pg	28–34 pg
$MCHC^{d} = \frac{\text{Hb (g/dL)}}{\text{Hct (\%)}} \times 100$	$\frac{14.2}{42.6} \times 100 = 33$ g/dL	32–36 g/dL

[a] Reference intervals listed in this table are for the adult population.
[b] If Hct is expressed in L/L, multiply by 1000 instead of 10.
[c] If Hb is expressed in g/L, do not multiply by 10.
[d] If Hct is expressed in L/L, do not multiply by 100; if Hct is expressed in L/L and Hb in g/L, divide results by 10 and do not multiply by 100.

the erythrocytes as they are packed during centrifugation in the centrifuged hematocrit sample. Likewise, the MCHC can also be affected by increased trapped plasma (e.g., sickle cell anemia). The manually calculated MCHC is lower than that obtained from the automated instrument. When the hematocrit is corrected for trapped plasma, the erythrocyte indices calculated from the centrifuged hematocrit agree with those obtained from automated instruments. The erythrocyte indices are used in the morphologic classification of anemias, defining normocytic, microcytic, and macrocytic anemias with the MCV (Chapters 10 and 11).

☑ CHECKPOINT 37-10

Given the following erythrocyte data, what is the expected erythrocyte morphology? Hemoglobin = 6.2 g/dL; hematocrit = 28%; erythrocyte count = 4.10×10^6/mcL

ERYTHROCYTE SEDIMENTATION RATE (ESR)

The ESR is a measurement of the rate at which the erythrocytes settle from the plasma. The sedimentation process consists of three phases. Phase 1 occurs within the first 5–10 minutes and represents the aggregation phase when erythrocytes form rouleaux. The second phase is the sedimentation phase when erythrocytes aggregate and the aggregates fall out of solution. The third phase is the packing phase in which the erythrocyte aggregates pack closely together at the bottom of the tube. The rate of erythrocyte settling depends on (1) the plasma's protein composition, (2) the erythrocytes' size and shape, and (3) the erythrocyte concentration.[31,32] Increasing levels of plasma proteins (primarily **acute phase reactants** such as C-reactive protein, fibrinogen, α globulins, or γ globulins whose synthesis rises early in inflammation or infection) result in a decrease of the ζ potential (the overall negative charge of the membrane) surrounding the erythrocytes. With a lower ζ potential, the erythrocytes are able to join together in rouleaux formation and settle from the plasma at a faster rate. Likewise, the erythrocytes' size and shape affect the rate of fall. Macrocytes settle faster than normal erythrocytes, and microcytes settle more slowly. Because of their irregular shape, poikilocytes are unable to form rouleaux and settle at a slower rate. The erythrocyte concentration directly affects the ESR (i.e., the higher the erythrocyte concentration, the lower the ESR). An anemic individual would appear to have an increased ESR.

The ESR is used to demonstrate the presence of inflammation and/or tissue destruction. It is a nonspecific test indicating tissue destruction/inflammation but not specifying the cause (i.e., disease state responsible). Refer to Table 37-10 ★ for a number of conditions associated with an elevated ESR.[33–35]

For ESR, the CLSI recommends the Westergren method in which EDTA-anticoagulated whole blood is diluted with 0.85% NaCl (see this chapter's Companion Resources for the detailed procedure). The diluted blood is aspirated into a calibrated Westergren pipet, and the cells are allowed to settle for a period of exactly 1 hour (Web Figure 37-6) at the end of which the distance in millimeters between the meniscus of the plasma and the top of the sedimented erythrocyte column is read. Potential sources of error in the modified

★ **TABLE 37-10** Conditions Associated with an Elevated ESR

Acute and chronic infections
Acute coronary syndrome
Acute ischemic stroke
Multiple myeloma
Osteomyelitis
Pelvic inflammatory disease
Polymyalgia rheumatica
Pregnancy
Pulmonary tuberculosis
Rheumatic fever
Rheumatoid arthritis
Systemic lupus erythematosus
Subacute bacterial endocarditis
Waldenstrom's macroglobulinemia

Westergren method for ESR are provided in Web Table 37-9. The reference interval for ESR varies with age and sex. For adult males and children, the reference interval is 0–10 mm/hr. The reference interval for adult females is 0–20 mm/hr.

Automated methods for determining ESR have been available since the 1990s. The principle of measurement for each instrument is an adaptation of the manual Westergren ESR method that allows determination of ESR in a shorter time period, typically 20–30 minutes. The automated methods convert the observed result to "mm/hr" so that reported results are in the conventional unit of measurement for the Westergren ESR. These methods have been verified to provide results comparable to those of the manual Westergren method.[36] The advantage of automated instruments is that they allow standardization of the procedure, increasing accuracy and reproducibility of the results.

One example of an automated ESR instrument is the VES-MATIC CUBE 80™ instrument (Diesse Diagnostica Senese, Milan, Italy). It is a closed-tube automated system that determines the ESR from whole blood samples in EDTA collection tubes.[37–39] The barcode-labeled collection tubes are placed in the instrument's analyzer rack of the loader/classifier module. A robotic system aligns collection tube's barcode label to the barcode reader to identify the sample and tests to be performed on it. The robotic system places the ESR samples in the test tube holder chain and samples are moved to the analytical module. Within the analytical module, the sample is mixed 15 times at a rotation of 120°. After the sample is mixed, it is transported to the reader point 1 for the "initial" optical reading. The chain movement within the analytical module allows the erythrocytes within the sample to settle for a 20-minute time period. At the end of this time period, the sample has reached the reader point 2 and the "final" optical reading is taken. The "initial" reading measures the initial height of the blood sample using a light emitting diode-based optical system, and the "final" reading measures the point between the erythrocyte layer and the plasma layer. The decrease in the height of the erythrocyte column is determined and used to mathematically derive the ESR in millimeters per hour.

The ESR-Auto Plus® instrument (Streck, Inc., Omaha, NE) is a random access instrument with 10 ESR tube positions.[40,41] The addition of the ESR barcode scanner integrates this instrument with a laboratory information system and creates a "walk-away" instrument. The instrument uses a special narrow bore vacuum tube containing 3.2% sodium citrate. When filled properly, blood is drawn to the 60 mm mark ± 5 mm. Alternatively, an EDTA-anticoagulated sample can be used. In this case, the proper amount of blood is transferred from the EDTA sample to the ESR vacuum tube. The vacuum tubes are thoroughly mixed using the ESR-657 mixer or manual mixing technique prior to placing them in the instrument. The vacuum tubes are held in a vertical position at ambient temperature. The instrument takes two measurements using infrared light, an "initial" reading at zero time to determine the fill level in the tube and a "final" reading after 30 minutes that reflects the erythrocyte sedimentation level. A mathematical formula is applied to the observed result to determine the ESR in millimeters per hour. Using this method, the measurement can be taken at 30 minutes because the majority of samples reach the "packing" phase of the erythrocyte sedimentation process by this time.

C-reactive protein (CRP) determinations by immunologic techniques such as nephelometry or enzyme-linked immunoassays have been introduced as a quantitative replacement for the ESR. CRP is an acute phase reactant that becomes elevated in the same conditions as the ESR. In fact, studies have demonstrated a rise in CRP levels prior to an elevation in the ESR.[42] Normal individuals have low levels, less than 5 mg/L, of CRP. With inflammation, CRP levels can rise to 20–500 mg/L within 8 hours of the acute inflammatory event.[43] High-sensitivity CRP assays (hsCRP) detect changes in CRP at the lower limit or below the linearity of the standard immunoassays for CRP determination. CRP measurements at these low levels are used in risk assessment for myocardial infarction and atherosclerosis.[44–46] Recent studies suggest hsCRP measurements can be used as a predictor of active infection in patients with systemic lupus erythematosus (SLE) allowing differentiation between active infection and disease flare.[47]

CRP and ESR results remain components in the classification criteria for rheumatoid arthritis. In the 2010 classification criteria, recommended by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR), CRP and ESR results are used in the acute phase reactant category to evaluate a patient's acute phase response.[48] Studies associated with the development of a new criteria showed that ESR and CRP results were predictive for various rheumatoid arthritis outcomes including response to methotrexate treatment.[49,50]

RETICULOCYTE COUNT

Reticulocytes are immature non-nucleated erythrocytes containing residual RNA (i.e., polychromatophilic erythrocytes). In the erythrocyte maturation sequence, the reticulocyte spends about 2 days in the bone marrow and 1 day in the peripheral blood. As the reticulocyte matures, the amount of RNA decreases. The quantitation of reticulocytes present in the peripheral blood provides a method of evaluating the bone marrow's erythropoietic activity. This evaluation is utilized in the differential diagnosis of anemias and in monitoring a patient's erythropoietic response to therapy (Chapters 5 and 11).[51–54]

Using a **supravital stain** (new methylene blue), residual ribosomal RNA is precipitated within the reticulocytes.[55] The detailed procedure is provided in this chapter's Companion Resources. A blood smear is prepared from the mixture of anticoagulated whole blood and supravital stain. The smear is examined microscopically using the oil immersion lens (1000× magnification) fitted with a field-restricted ocular (Figure 37-13 ■). An erythrocyte containing two or more particles of blue-stained material is a reticulocyte (Figure 37-14 ■). The number of reticulocytes is expressed as a percentage of the total number of erythrocytes counted (Web Figure 37-7).

Other erythrocyte inclusions (Pappenheimer bodies, Howell-Jolly bodies, and Heinz bodies; Chapter 10) can also be stained with new methylene blue and must be distinguished from reticulocytes. Heinz bodies and Howell-Jolly bodies are distinguished from precipitated reticulum by their shape and staining characteristics. Heinz bodies appear as light blue–green inclusions located at the periphery of the erythrocyte. Howell-Jolly bodies are usually one or two round, deep-purple staining inclusions and are visible on

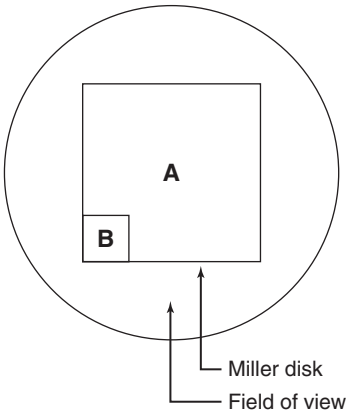


FIGURE 37-13 Miller disk. The number of reticulocytes in square A divided by the total number of RBCs counted in square B times 9 (to correct for the difference between the area of the two squares) times 100 equals the reticulocyte count as a percentage. See Web Figure 37-7 for example.

A = reticulocyte counting area; B = erythrocyte counting area (area of the B square is one-ninth the area of the A square)

Romanowsky stains. Pappenheimer bodies are indistinguishable from reticulum of reticulocytes. If Pappenheimer bodies are suspected, a Prussian blue iron stain (Chapter 38) should be performed to verify their presence. Reticulum does not stain with the Prussian blue iron stain.

Misinterpretations can occur when only the percentage of reticulocytes present in the peripheral blood is reported because the reticulocyte percentage is a relative number and depends on the total number of erythrocytes present in the peripheral blood. If the total erythrocyte count is decreased, the reticulocyte percentage does not accurately reflect the bone marrow's production of new erythrocytes. The absolute reticulocyte count, corrected reticulocyte count, and the reticulocyte production index can be used to avoid these interpretation errors. These calculations are discussed in Chapters 10 and 11.

Automation is becoming a more popular method for determining the reticulocyte count. The new generation of automated hematology instruments is capable of performing absolute reticulocyte counts and other useful parameters. A thorough discussion can be found in Chapter 39.

☑ CHECKPOINT 37-11

Morphologic evaluation of a Wright-stained peripheral blood smear reveals the presence of Pappenheimer bodies. How does this affect a reticulocyte count to be performed on the same blood sample? How would you confirm the presence of Pappenheimer bodies?

SOLUBILITY TEST FOR HEMOGLOBIN S

The solubility test is the most commonly used screening test for the presence of hemoglobin S (Chapters 6 and 13). It is based on the relative insolubility of hemoglobin S when combined with a reducing agent (sodium dithionite or sodium hydrosulfite).[56] Anticoagulated whole blood is mixed with a lysing agent (saponin) and reducing agent. This solution releases the hemoglobin from the erythrocytes and reduces it. If HbS is present, it forms crystals and gives a turbid appearance to the solution (Figure 37-15 ■). A transparent solution is seen with other hemoglobins that are more soluble in the reducing agent (see this chapter's Companion Resources for the detailed procedure).

The solubility test does not differentiate hemoglobin S disease from hemoglobin S trait. A hemoglobin electrophoresis procedure should be performed to differentiate these two states. In addition, several abnormal hemoglobin variants can cause sickling and give

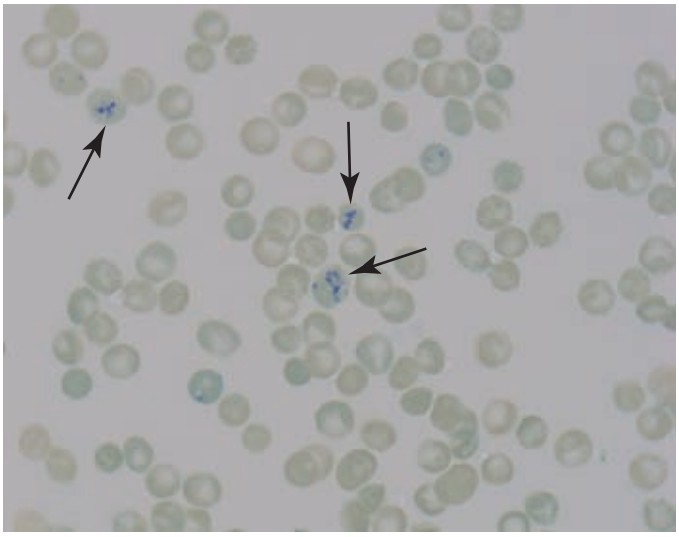


FIGURE 37-14 Reticulocytes identified by new methylene blue stain. The reticulocytes are the cells (arrows) containing bluish purple particulate inclusions (1000× original magnification).

FIGURE 37-15 Sodium dithionite tube test. Negative results are indicated by the clear solution where the black lines on the reader scale are visible through the test solution (right). Positive results are shown as a turbid solution where the reader scale is not visible through the test solution (left).

a positive solubility test. These variants include HbC Harlem, HbS Travis, and HbC Ziguinchor. High-pressure liquid chromatography or **isoelectric focusing** is used to differentiate these variants from HbS.

PART II

REFLEX HEMATOLOGY PROCEDURES

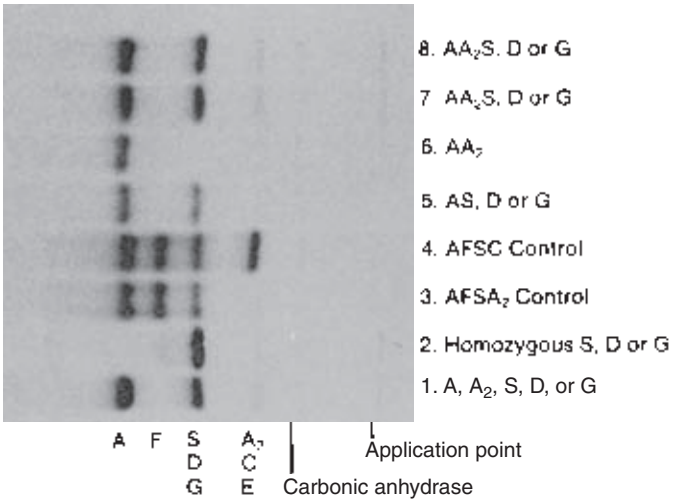
Reflex tests can be performed when a screening test reveals abnormal results. Some laboratories create algorithms or flow charts to help the physician determine which reflex tests should be ordered based on the abnormal screening test(s). The reflex tests help to identify the hematologic abnormality or other pathology and to determine a diagnosis as well as treatment.

HEMOGLOBIN ELECTROPHORESIS

Electrophoresis is the movement of charged molecules in an electric field. Use of this procedure can detect and preliminarily identify hemoglobinopathies and thalassemias (Chapters 13 and 14).[57] In hemoglobin electrophoresis, hemoglobin A (adult hemoglobin) takes on a net negative charge at an alkaline pH and moves the farthest toward the anode (positive electrode). Many abnormal hemoglobin variants have altered charges because of single amino acid substitutions within their globin chains (Chapter 13). This change in the degree of the negative charge allows for the separation of the majority of abnormal hemoglobin variants from hemoglobin A at an alkaline pH.[58,59] The electrophoretic patterns and hemoglobin percentages of the unknown (patient) samples are compared with those of the control samples; one control contains HbA, -F, -S, and -C, and the other control contains HbA and HbA_2.

In the hemoglobin electrophoresis procedure, EDTA-anticoagulated whole blood is centrifuged to obtain packed erythrocytes. The erythrocytes are washed, and a hemolysate is obtained by lysing them with a hemolysate reagent (e.g., 0.005 M EDTA, 0.175% saponin, and 0.07% potassium cyanide). The hemolysate (i.e., patient and controls) is applied to an agarose gel, which is then placed in the electrophoresis chamber. An alkaline buffer, pH 8.6, permits the flow of electrons from the cathode to the anode within the chamber, alters the charge of the hemoglobin molecules based on their amino acid composition, and allows for the migration and separation of the hemoglobin molecules based on the strength of their negative charge. The hemoglobin molecules are allowed to electrophorese (migrate) for 25 minutes. Following the electrophoresis, the agarose gel is stained with acid blue, an anionic dye, destained to remove excess dye, and dried. The hemoglobin gel can be examined visually to determine each sample's hemoglobin electrophoretic pattern or with a **densitometer** using 595 nm filter to determine the percentage of hemoglobin present in each band or region. Newer hemoglobin/protein electrophoresis systems use a flat-bed scanner and specific computer program to determine the percentage of hemoglobin present in each band or region and generate the sample's electrophoretic pattern.

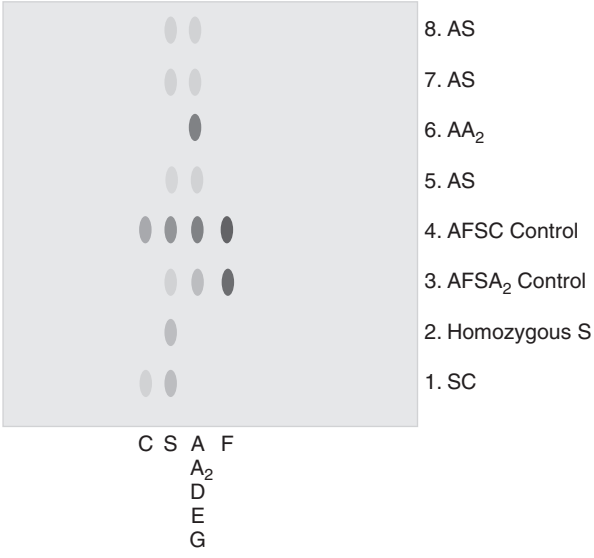
Alkaline hemoglobin electrophoresis allows for the separation of HbA, -F, -S, and -C into distinct bands (Figure 37-16 ■). However, other abnormal hemoglobin variants have the same electrophoretic mobility as HbS and HbC. HbD and HbG have the same mobility as HbS whereas HbE and HbO_{Arab} have the same mobility as HbC. Thus, additional tests must be used to confirm the presence of these abnormal hemoglobin variants. The solubility test can be used to confirm HbS, and citrate agar electrophoresis can be used to confirm the presence of HbS and HbC.



■ **FIGURE 37-16** Electrophoretic mobilities of hemoglobins on cellulose acetate at pH 8.6.

A, A_2, D, G, S, F, C, E = hemoglobin variants
Courtesy of Helena Laboratories, Beaumont, TX.

Citrate agar electrophoresis at pH 6.2 separates HbA, -F, -S, -C into distinct bands (Figure 37-17 ■). Because no other hemoglobin variants travel with HbS and HbC on citrate agar, it is used to confirm their presence.[60] It is also useful in the differentiation of other hemoglobin variants that travel with HbS and HbC on alkaline hemoglobin electrophoresis; these hemoglobin variants (HbD, HbG, HbE, and HbO_{Arab}) have the same mobility as HbA on citrate agar. Rare



■ **FIGURE 37-17** Electrophoretic mobilities of hemoglobins on citrate agar at pH 6.2, showing separation of hemoglobins S and C from other hemoglobins.

A, A_2, S, F, C, D, E, G = hemoglobin variants

☑ CHECKPOINT 37-12

What is the basis for hemoglobin separation in the hemoglobin electrophoresis procedures?

abnormal hemoglobin variants can be positively identified with DNA analysis of the globin genes (Chapter 42).

QUANTITATION OF HEMOGLOBIN A_2

HbA_2 is a normal adult hemoglobin present in small amounts (up to 3.5%). Increased amounts of HbA_2 are characteristic of β thalassemia minor. Therefore, the quantitation of HbA_2 is useful in making a presumptive diagnosis. Slight increases in HbA_2 concentration also have been noted in persons with HbS trait, HbS disease, unstable hemoglobin variants, or megaloblastic anemia. Decreased HbA_2 concentrations can be seen in iron-deficiency anemia and α thalassemia.

HbA_2 can be quantitated with anion exchange **column chromatography**. The anion exchange resin is a preparation of cellulose covalently coupled to small positively charged molecules. Thus, the anion exchange resin attracts negatively charged molecules. Hemoglobins, like other proteins, contain positive and negative charges because of the ionizing properties of their component amino acids. In the anion exchange chromatography of HbA_2, the ionic strength of the buffer and the pH levels are controlled to cause different hemoglobins to possess different net negative charges.[61] These negatively charged proteins are attracted to the positively charged cellulose and bind accordingly. Following binding, the hemoglobins are removed selectively from the resin by altering the pH or ionic strength of the elution buffer. Due to the solubility of HbA_2 in the elution buffer, HbA_2 is eluted from the resin as the elution buffer moves through the column. The resin retains the other normal, and most abnormal, hemoglobins. The percentage of HbA_2 is determined by comparing the absorbance of the HbA_2 fraction to the absorbance of the total hemoglobin fraction at 415 nm using a spectrophotometer. The reference interval for HbA_2 is 1.8–3.5%.

Values between 3.5–8% are considered indicative of β thalassemia trait (Chapter 14). Values above 8% indicate the presence of additional hemoglobin variants such as S, C, E, O, D, and S-G hybrid, which elute with HbA_2 (Chapter 13). HbA_2 cannot be differentiated from several abnormal hemoglobin variants such as hemoglobins C, E, and O, which have a net electrical charge similar to HbA_2 at pH 8.6. If abnormal hemoglobin variants are suspected, other hemoglobin electrophoretic techniques or DNA analysis of globin chains should be performed to confirm their presence. HbA_2 levels can be normal when iron-deficiency anemia coexists with β-thalassemia minor. In this situation, HbA_2 levels must be considered with family history, laboratory data including serum ferritin, serum iron, total iron-binding capacity, red cell morphology, Hb, Hct, and MCV.

Other techniques can be used to quantitate HbA_2, including high-pressure liquid chromatography and capillary electrophoresis. The Bio-Rad VARIANT™ II hemoglobin instrument measures HbA_{1C} levels for glycemic control in diabetic patients and can be used to quantitate HbA_2, HbF, and certain hemoglobin variants.[62–64] For the quantitation of these hemoglobins, this fully automated instrument requires a separate cation-exchange column, specific sodium phosphate buffers, and elution program (i.e., Beta-Thal Short program). Within the instrument, a portion of EDTA-anticoagulated blood sample is hemolyzed and diluted and then injected into the cation exchange column. The hemoglobins are separated using a gradient of sodium phosphate buffers of increasing ionic strength for a 6.5-minute elution. The eluted hemoglobins pass through a flow cell where the absorbance is measured at 415 nm with correction at 690 nm. The eluted hemoglobin is identified by its retention time. For example, the retention time of HbA_2 is 3.3–3.9 minutes. The concentration of the eluted hemoglobin is determined from its absorbance measurement.

The Sebia CAPILLARYS™ 2 instrument is a fully automated system that separates hemoglobin by capillary electrophoresis and quantitates each hemoglobin based on its absorbance reading by spectrophotometry.[65,66] Thus, the concentration of HbA_2 can be determined. In capillary electrophoresis, separation of hemoglobins including HbA_2, HbF, HbS, and other hemoglobin variants within a given sample is achieved by introducing a hemolysate derived from the packed erythrocytes of the EDTA-anticoagulated sample to an alkaline buffer (pH 9.4) that is running through a narrow-bored capillary under high voltage. As the hemoglobins leave the capillary, the absorbance reading of each separated hemoglobin is determined at 415 nm. These data are used to generate a hemoglobin electrophoresis pattern for the sample and determine the concentration of each hemoglobin including HbA_2 as a percentage of the total hemoglobin.

ACID ELUTION FOR HEMOGLOBIN F

The acid elution test for fetal hemoglobin (HbF) can be used in the differentiation of hereditary persistence of fetal hemoglobin (HPFH) from other conditions associated with high levels of HbF (Chapters 13 and 14). HPFH is characterized by an even or uniform distribution of HbF within the erythrocytes, whereas other conditions with high HbF levels are characterized by an uneven or nonuniform distribution of HbF within the erythrocytes. Conditions such as β thalassemia minor, β thalassemia major, sickle cell anemia, hereditary spherocytosis, and aplastic anemia are associated with high levels of HbF but nonuniform distribution. This test can also be used to detect the presence of fetal cells in the maternal circulation (fetal–maternal bleed) during problem pregnancies.

In an acid solution, all hemoglobins except hemoglobin F (fetal hemoglobin) are eluted from the erythrocytes.[67] Blood smears are fixed in 80% ethanol and then incubated in the elution buffer, citrate-phosphate (pH 3.3). The slide is stained with acid hematoxylin and counterstained with eosin. The slide is observed microscopically using the oil immersion lens to determine the distribution of HbF within the erythrocytes and the percentage of HbF-containing erythrocytes. Erythrocytes containing fetal hemoglobin stain bright pink or red with the eosin B stain (Figure 37-18 ■). The remaining erythrocytes appear as pale ghosts. Intermediate erythrocytes (pink colored but not intense) are sometimes seen. The acid hematoxylin stains the leukocyte's nuclei a faint gray-purple for differentiation.

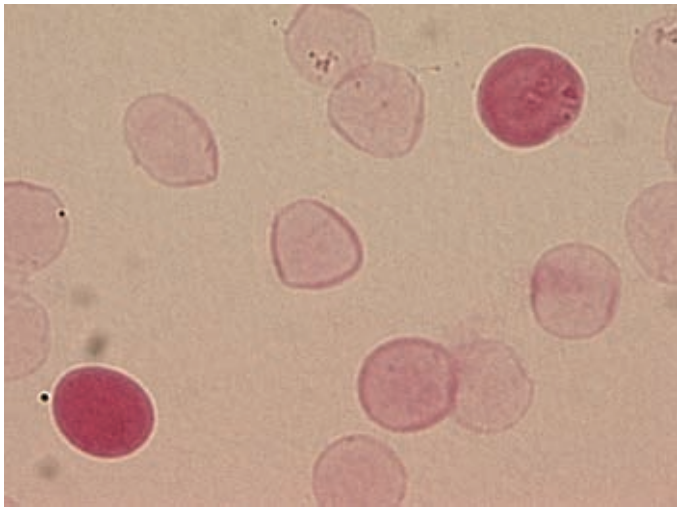


FIGURE 37-18 Acid elution test for determination of hemoglobin F. Erythrocytes containing hemoglobin F appear as bright pink staining cells. The light staining cells are erythrocytes that contain adult hemoglobins.

QUANTITATION OF HEMOGLOBIN F

Hemoglobin F is the predominant hemoglobin in the fetus and newborn but makes up less than 2% of adults' total hemoglobin. Hemoglobin F can be found in increased amounts in some types of anemia such as thalassemia.

Alkali Denaturation

HbF resists denaturation by strong alkali solutions, but other hemoglobins are not resistant. The addition of a strong alkali solution (1.27 M NaOH) to a hemolysate containing a known concentration of hemoglobin results in hemoglobin denaturation.[68] Adding a saturated solution of ammonium sulfate stops the denaturation process. Ammonium sulfate lowers the pH of the reaction mixture and precipitates all denatured hemoglobins. After filtration, the concentration of the remaining alkali-resistant hemoglobin is determined. The alkali-resistant hemoglobin is expressed as a percentage of the total hemoglobin concentration. The cyanmethemoglobin method determines the hemoglobin concentrations (alkali resistant and total). The general reference interval for an adult is less than 2%.

Other Methods

In the clinical laboratory, HbF is typically determined by high-pressure liquid chromatography as discussed previously with HbA_2 quantitation or by flow cytometry. In flow cytometry, the cells are permeabilized to allow entry of the fluorescent-conjugated monoclonal antibody directed against HbF into the cell for recognition of HbF. The advantage of flow cytometry is that an increased number of cells (~10,000) are evaluated, thus improving the result's accuracy. In addition, flow cytometry allows the determination of HbF concentration within individual cells, and with the addition of thiazole orange, the differentiation of cells containing HbF—such as mature erythrocytes, polychromatophilic erythrocytes or reticulocytes, and nucleated erythrocytes—can be made, which can be useful in evaluating certain hematologic diseases (e.g., β-thalassemia).[69]

☑ CHECKPOINT 37-13

What are the advantages of using flow cytometry to quantitate HbF?

HEAT DENATURATION TEST FOR UNSTABLE HEMOGLOBIN

Unstable hemoglobins are hemoglobin variants that result from a variety of amino acid substitutions or deletions affecting the hemoglobin molecule's intramolecular interactions (Chapter 13). These hemoglobin variants are susceptible to spontaneous denaturation resulting in Heinz body formation and erythrocyte hemolysis. In the laboratory, blood samples can be manipulated so that unstable hemoglobins become insoluble and form flocculent precipitate at higher temperatures (50°C), whereas normal hemoglobin remains soluble.[70,71] The cyanmethemoglobin method is used to determine the hemoglobin concentrations for heated and unheated fractions. The concentration of unstable hemoglobin is expressed as a percentage of the total hemoglobin concentration (unheated fraction). Low concentrations of unstable hemoglobin result in false negative results.

Unstable hemoglobins can cause congenital nonspherocytic hemolytic anemias (Chapter 13). Examples of unstable hemoglobins are hemoglobin Koln, hemoglobin Hammersmith, hemoglobin Zurich, hemoglobin Seattle, and hemoglobin Bristol. A positive heat denaturation test should be confirmed by other test methods that identify unstable hemoglobins, such as tests for erythrocyte inclusions and the **isopropanol precipitation** test. The specific unstable hemoglobin is identified by DNA sequence analysis to determine the hemoglobin mutation followed by HPLC and tandem mass spectrometry analysis to confirm the specific amino acid substitution.

HEINZ BODY STAIN

Heinz bodies represent denatured hemoglobin inclusions. These inclusions are usually round or oval, appear refractile, and tend to locate adjacent to the erythrocyte membrane. Heinz bodies are visible only on supravital-stained smears and are not visible on Wright-stained smears. Heinz bodies can be present in glucose-6-phosphate dehydrogenase deficiency and related enzyme disorders when the individual is exposed to oxidizing agents such as primaquine or sulfanilamide (Chapter 18). In addition, they can be found in individuals with unstable hemoglobins or thalassemias. Heinz bodies are occasionally found in senescent erythrocytes of normal individuals.

A specific dye for Heinz bodies is brilliant green.[72] To visualize Heinz bodies, EDTA-anticoagulated whole blood is first mixed with 0.5% neutral red. The mixture is counterstained with 0.5% brilliant green. Several thick smears are prepared from the final mixture. The

smears are observed microscopically for the presence of Heinz bodies. Heinz bodies stain green, reticulocytes and Howell-Jolly bodies stain a deep red, and erythrocytes stain light red. The percentage of erythrocytes containing Heinz bodies can be determined by counting the number of erythrocytes containing Heinz bodies within 500 erythrocytes.

The specificity of the brilliant green dye for Heinz bodies eliminates problems that arise with the use of other supravital stains such as methyl violet or crystal violet. With these other supravital stains, Howell-Jolly bodies, basophilic stippling, and reticulum stain the same as Heinz bodies, often leading to difficulties in interpreting the stain.

OSMOTIC FRAGILITY TEST

In the osmotic fragility test, anticoagulated whole blood is added to increasingly hypotonic solutions of buffered sodium chloride (0.85–0.00%), and the solutions incubate for 20 minutes at room temperature.[73] The amount of hemolysis at each concentration is determined by measuring the absorbance of the **supernatants** spectrophotometrically (see this chapter's Companion Resources for the detailed procedure and Web Table 37-10). An osmotic fragility graph is prepared by plotting the percentage of hemolysis for each solution against its concentration, and the results are compared with a normal control. In normal individuals, an almost symmetrical sigmoid-shaped curve is obtained (Web Figure 37-8). Normal erythrocytes begin to hemolyze around 0.50% sodium chloride (NaCl) concentration, and hemolysis is complete at 0.30% NaCl. The normal values for osmotic fragility with each sodium concentration are given in Table I in the inside book cover.

In the osmotic fragility test, spherocytes with a decreased surface-area-to-volume ratio have a limited ability to expand in hypotonic solutions. They lyse at higher concentrations of sodium chloride than normal biconcave erythrocytes and are said to have an increased osmotic fragility.[74] Target cells or sickle cells have a large surface-area-to-volume ratio. This increased surface-area-to-volume ratio translates into an increased ability to expand in hypotonic solutions. These cells lyse at lower concentrations of sodium chloride than normal cells and are said to have a decreased osmotic fragility.

An increased osmotic fragility is associated with hemolytic anemias in which spherocytes are present, for example, hereditary spherocytosis (Chapter 17). Conditions associated with a decreased osmotic fragility include thalassemia, sickle cell anemia, and those conditions in which target cells are observed. Figure 37-19

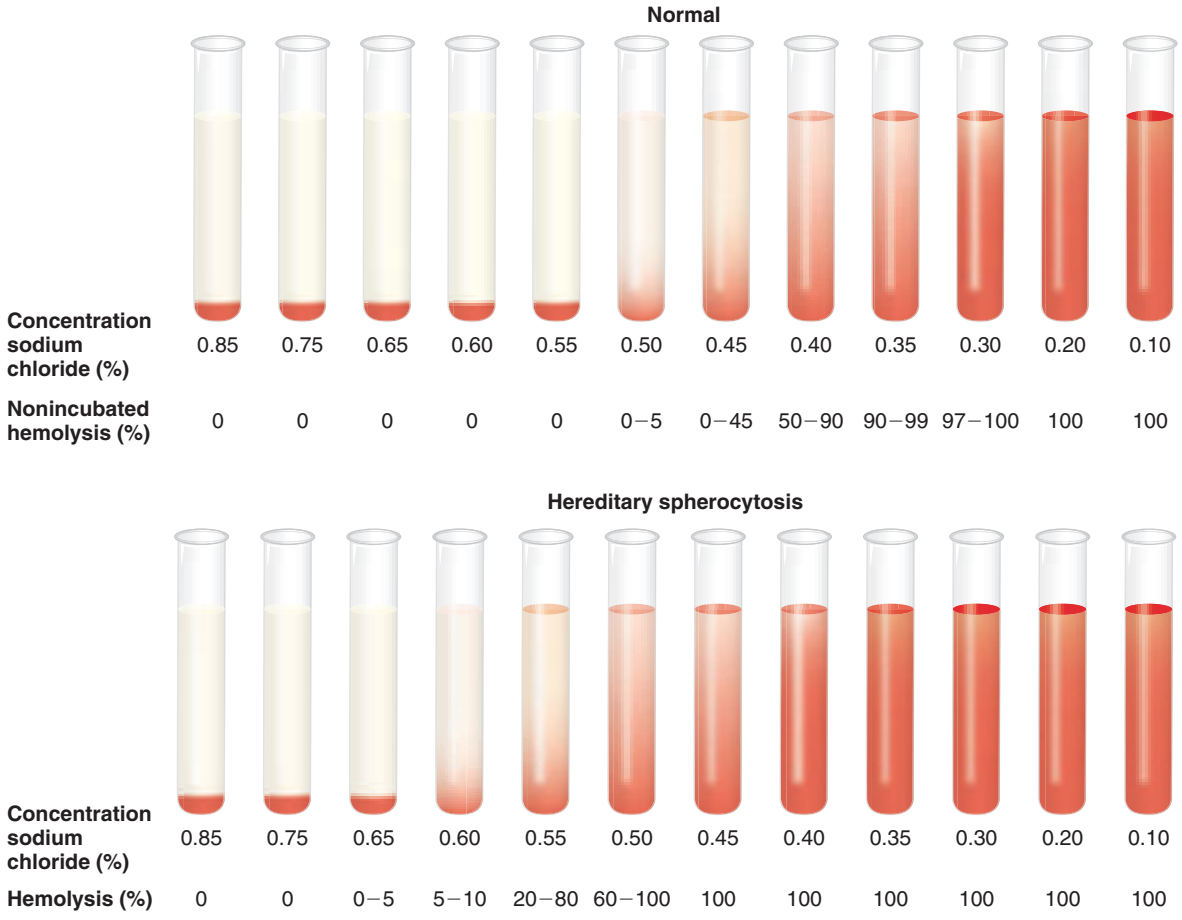


FIGURE 37-19 The osmotic fragility test demonstrates the increased osmotic lysis of spherocytes in a hypotonic medium compared with normal erythrocytes.

Clear supernatant = no hemolysis; pinkish supernatant = partial hemolysis; red supernatant = complete hemolysis

depicts the increased osmotic lysis of spherocytes in a hypotonic medium.

An incubated osmotic fragility test is performed to identify patients with mild hereditary spherocytosis in which the standard osmotic fragility test is normal. In this test, patient and control blood samples incubate at 37°C under sterile conditions for 24 hours Then, the osmotic fragility test is performed on those samples. A significantly increased osmotic fragility after incubation is characteristic of hereditary spherocytosis.

☑ CHECKPOINT 37-14

A patient's osmotic fragility test shows beginning hemolysis at 0.60% NaCl and complete hemolysis at 0.50% NaCl. How should these results be interpreted?

DONATH-LANDSTEINER TEST FOR PAROXYSMAL COLD HEMOGLOBINURIA (PCH)

The Donath-Landsteiner test is a screening test for PCH,[75] which is characterized by the presence of the Donath-Landsteiner antibody, a **biphasic antibody** with anti-P specificity (Chapter 19). This IgG antibody is capable of activating complement resulting in hemolysis. The Donath-Landsteiner test should be performed when an individual presents with hemoglobinuria and a positive direct antiglobulin test due to presence of complement (C3) only with no evidence of autoantibody activity in the serum. In this procedure, a series of test tubes is set up to detect the biphasic nature of complement activation (Table 37-11 ★). Normal serum serves as a source of complement because individuals with PCH can express low levels of complement. The 50% suspension of group O erythrocytes with P antigen serves as the antibody receptor. Following the appropriate incubation schedule, the tubes are centrifuged and observed for hemolysis. If anti-P present in the patient's serum, it binds to the erythrocyte's P antigen during the incubation time in the melting ice bath. During the second incubation time at 37°C, the antibody dissociates from the erythrocytes, and complement is activated, leading to hemolysis. Therefore, the test is considered positive for PCH if tubes A1 and/or A2 demonstrate hemolysis and the remaining tubes have none. Proper sample collection and processing are essential to this test's outcome. The patient's blood should be allowed to clot at 37°C and the serum separated at this temperature to avoid cold autoabsorption and loss of antibody prior to testing.

ERYTHROPOIETIN

Erythropoietin (EPO) levels are determined by enzyme-linked immunosorbent assay (ELISA). This procedure uses microtiter plate wells coated with a monoclonal mouse antihuman antibody directed against EPO.[76] This antibody represents the capture antibody because it binds EPO from the serum, either patient, control, or standard. Following a washing step, the wells are incubated with a polyclonal rabbit anti-EPO that is enzyme-labeled with horseradish peroxidase. This second antibody binds to the initial antigen–antibody complex. Hence, EPO is sandwiched between two specific antibodies (Web Figure 37-9). A substrate specific for the enzyme label (i.e., tetramethylbenzidine) is added to the microtiter plate wells, and a colorimetric reaction occurs. The absorbance of each microtiter well is determined using a microtiter plate reader, an adaptation of a spectrophotometer. The amount of absorbance measured in a given microtiter well is directly proportional to the concentration of EPO in the sample. The control and patient results are determined from a reference (standard) curve that is prepared using known concentrations of EPO. The general reference interval for serum EPO is 3.3–16.6 mIU/mL.

Measurement of EPO levels is useful in diagnosing certain anemias and polycythemia.[77] For example, secondary polycythemias such as chronic obstructive pulmonary disease or cyanotic heart disease are associated with elevated levels of EPO, whereas the myeloproliferative disorder polycythemia vera is associated with normal to low EPO levels (Chapter 24). Decreased levels of EPO are observed in anemia of renal failure, anemia of chronic disease (also called anemia of chronic inflammation), and anemia of hypothyroidism (Chapters 12 and 16).

★ **TABLE 37-11** Schematic Outline of Donath-Landsteiner Test Procedure for Detecting PCH

Incubation Set	Tube 1	Tube 2	Tube 3		Incubation Protocol
A	Patient's serum	Patient's serum	—	→	30 minutes in melting ice bath followed by
	—	Normal serum	Normal serum		60 minutes at 37°C
	50% group O cells	50% group O cells	50% group O cells		
B	Patient's serum	Patient's serum	—	→	90 minutes in melting ice bath
	—	Normal serum	Normal serum		
	50% group O cells	50% group O cells	50% group O cells		
C	Patient's serum	Patient's serum	—	→	90 minutes @ 37°C
	—	Normal serum	Normal serum		
	50% group O cells	50% group O cells	50% group O cells		

Paroxysmal cold hemoglobinuria (PCH) is indicated if hemolysis occurs in tubes A1 and/or A2 and remaining tubes have no hemolysis. Tubes A3, B3, and C3 represent controls for normal serum complement source and should not demonstrate hemolysis. Tubes B1 and B2 represent controls for the presence of cold-reacting antibodies, and tubes C1 and C2 represent controls for the presence of warm-reacting antibodies. Including these controls to eliminate possible false positive interpretations is important.

SOLUBLE TRANSFERRIN RECEPTOR

Soluble transferrin receptor (sTfR) represents a truncated form of the membrane transferrin receptor that is normally found on the surface of cells that require iron. An ELISA procedure determines sTfR levels. This procedure uses microtiter plate wells coated with a monoclonal antihuman antibody directed against sTfR.[78] This antibody represents the capture antibody because it binds sTfR from the serum from either patient, control, or standard. Following a washing step, the wells are incubated with a second monoclonal anti-sTfR that is enzyme labeled with horseradish peroxidase. This second antibody binds to the initial antigen–antibody complex. Hence, sTfR is sandwiched between two specific antibodies (see Web Figure 37-9 for comparison). A substrate specific for the enzyme label is added to the microtiter plate wells, and a colorimetric reaction occurs. The absorbance of each microtiter well is determined using a microtiter plate reader; the amount of absorbance measured in a given microtiter well is directly proportional to the concentration of sTfR in the sample. The control and patient results are determined from a reference (standard) curve that is prepared using known concentrations of sTfR. The general reference interval for serum sTfR is 8.7–28.1 nmol/L.

Measurement of sTfR levels is useful in differentiating the diagnosis of iron-deficiency anemia from anemia of chronic inflammation because sTfR levels are elevated in iron-deficiency anemia but are normal in anemia of chronic inflammation (Chapter 12).

☑ CHECKPOINT 37-15

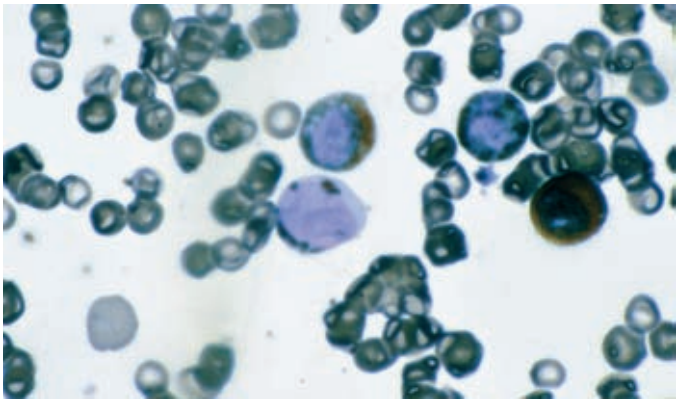
A patient was recently diagnosed with a hypochromic, microcytic anemia. Additional laboratory testing revealed EPO 1.5 mIU/mL and sTfR 15.8 nmol/L. Which anemia is consistent with these results?

CYTOCHEMICAL STAINS

Our expanding knowledge of neoplastic disorders,routine use of immunophenotyping by flow cytometry, and molecular testing to identify specific genetic mutations have largely replaced the need for cytochemical stains for classifying these disorders. However, there remain instances in which the results of cytochemical stains provide useful adjunct information for classifying neoplastic disorders. These cytochemical stains are discussed in this section.

Myeloperoxidase

Myeloperoxidase (MPO) is an enzyme capable of catalyzing the oxidation of substances by hydrogen peroxide. Fresh smears are used to ensure optimal reactivity of the enzyme. The methods using 3,3-diaminobenzidine as the color reagent with Giemsa counterstain or using 3-amino-9-ethylcarbazole provide satisfactory results.[79] This enzyme is present in primary granules of neutrophils, eosinophils, and monocytes. An insoluble dark brown reaction product identifies the sites of MPO activity (Figure 37-20 ■). MPO is the most sensitive and specific stain for granulocytes, which stain intensely. Monocytes stain less intensely than neutrophils. Lymphocytes do not exhibit MPO activity.

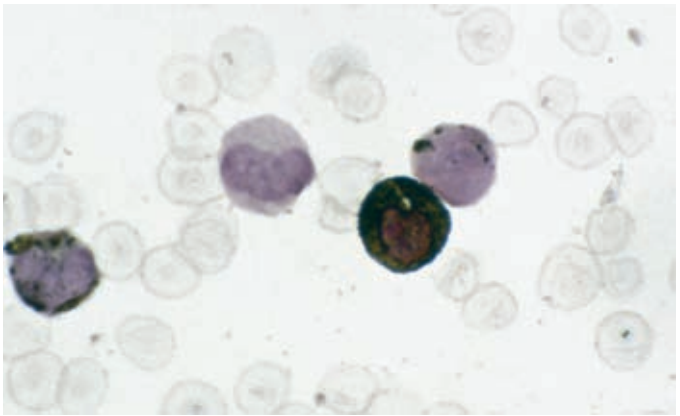


■ **FIGURE 37-20** Blasts of AML showing a positive myeloperoxidase stain (brown-black color in cytoplasm) (bone marrow; MPO stain; 1000× magnification).

Therefore, MPO is useful in differentiating acute myelogenous leukemia (AML) from acute lymphocytic leukemia (ALL) and subgrouping of the AMLs (Table 37-12 ★; Web Table 37-11). A positive reaction is seen in myeloblasts, and weak staining is rarely seen in monoblasts. Leukemic blasts that are negative for MPO can represent lymphoblasts, AML (minimally differentiated), monoblasts, erythroblasts, and megakaryoblasts, or undifferentiated leukemia.[80] The presence of many mature neutrophils with negative MPO staining can indicate an MPO deficiency.

Sudan Black B

Sudan black B (SBB) is a diazo dye that stains phospholipids, neutral fats, and sterols.[81] Cellular components containing lipids stain brown-black (Figure 37-21 ■). SBB stains phospholipids in membranes of primary and secondary granules of the granulocytic series. Auer rods have a rich phospholipid membrane that the SBB stain identifies; its results parallel those seen with the MPO stain in myeloblasts and monoblasts (Table 37-12; Web Table 37-11). Lymphoblasts do not stain with SBB (rare cases are weakly positive). It is useful when fresh samples are not available for the MPO stain and in unusual cases when myeloblasts have an acquired deficiency of MPO. Therefore, SBB is



■ **FIGURE 37-21** Blasts of AML showing a positive Sudan black B stain (brown-black color in cytoplasm) (bone marrow; SBB stain; 1000× magnification).

★ **TABLE 37-12** Cytochemical Features of Acute Myelogenous Leukemia (AML) Subgroups

Subgroup	Myeloperoxidase or Sudan Black B	Chloroacetate (specific) Esterase	Nonspecific Esterase
AML-minimally differentiated	Negative	Negative	Negative
AML-without maturation	Positive	Positive	Negative
AML-with maturation	Positive	Positive	Negative
AML-with t(15;17) or APL	Positive	Positive	Negative
AML-myelomonocytic	Positive	Positive	Positive[a]
AML-monoblastic/monocytic	Negative	Negative	Positive[a]
Acute erythroid leukemia	Positive[b]	Negative	Positive or negative
AML-megakaryoblastic	Negative	Negative	Strongly positive when acetate is used as a substrate

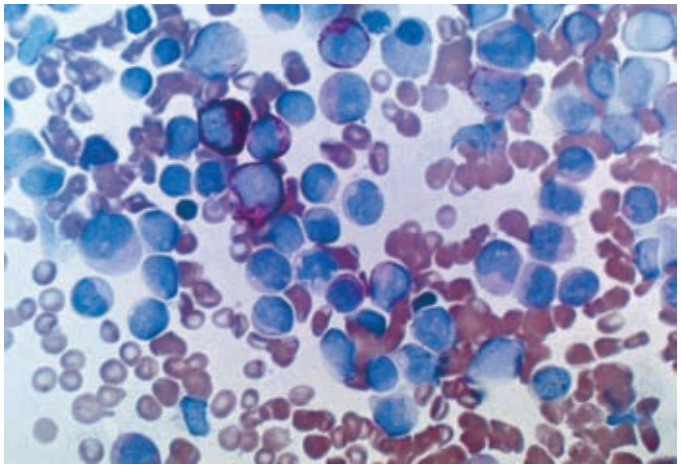
APL = acute promyelocytic leukemia

[a]Monocytic component is positive, and the staining can be inhibited by sodium fluoride incubation. About 20% of AML-monoblastic can be negative for nonspecific esterase.
[b]Myeloblasts in AML-erythroid are positive.

useful in differentiating AML from ALL. Because of its fat solubility, SBB also can stain marrow fat and some cytoplasmic vacuoles of Burkitt lymphoma cells.[82]

Chloroacetate Esterase

Several cytochemical methods are available for specific esterase on smears. Chloroacetate esterase (also called *specific esterase*) activity is detected by incubating the sample in naphthol AS-D chloroacetate at an acid pH.[83] The esterase activity causes enzymatic hydrolysis of ester linkages and liberates free naphthol compounds. The liberated naphthol immediately couples with a diazonium salt (e.g., fast red violet) forming an insoluble, visible pigment at the site of enzyme activity (Figure 37-22 ■). Naphthol AS-D chloroacetate is considered specific for the granulocytic series and is present in mast cells. The sites of enzyme activity show bright red granulation when fast red violet is used. Enzyme activity is weak or absent in monocytes and lymphocytes. Neoplastic eosinophils of acute myelomonoblastic leukemia rarely are positive for chloroacetate esterase. The naphthol AS-D chloroacetate is less sensitive than MPO and SBB, but positive results parallel those seen with the MPO and SBB stains. Therefore, it is useful in differentiating AML from ALL (Table 37-12; Web Table 37-11). A combination of chloroacetate esterase and cyanide-resistant peroxidase stains is also helpful in diagnosing acute eosinophilic leukemia, which is characteristically negative for chloroacetate esterase and positive for cyanide-resistant peroxidase.[80]



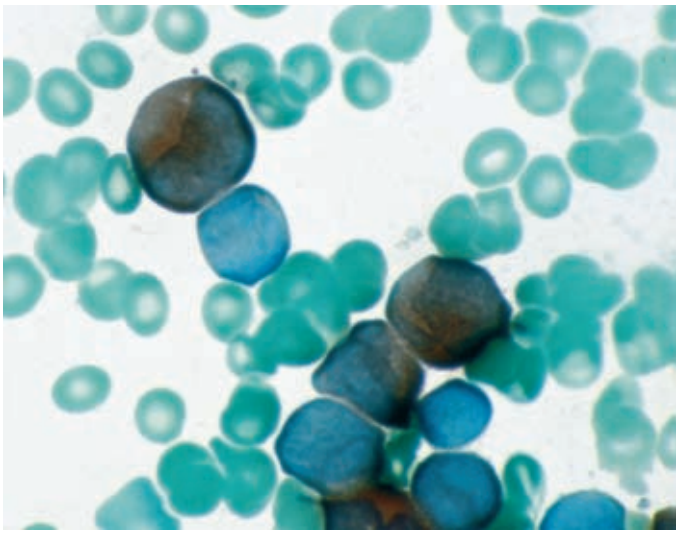
■ **FIGURE 37-22** Blasts of AML showing a positive specific esterase stain (red-magenta color in cytoplasm) (bone marrow; specific esterase stain; 500× magnification).

α-Naphthyl Esterase (Nonspecific Esterase)

α-Naphthyl esterase activity is found primarily in monocytes and macrophages using either α-naphthyl acetate or α-naphthyl butyrate as substrate. α-Naphthyl acetate esterase (ANAE) activity is also present in plasma cells, megakaryocytes, hairy cells, and T lymphocytes (dotlike pattern). Similarly, α-naphthyl butyrate esterase activity is also present in megakaryocytes (but less intense than ANAE activity) and helper T lymphocytes. The sites of enzyme activity show black granulation and are detected by incubating the sample in α-naphthyl acetate or butyrate at an alkaline pH (Figure 37-23 ■). The esterase activity causes enzymatic hydrolysis of ester linkages to occur and liberates free naphthol compounds. The liberated naphthol immediately couples with a diazonium salt (e.g., fast blue RR), forming an insoluble, visible pigment at the site of enzyme activity.[83]

Granulocytes are usually negative but can show occasional activity. In addition, enzyme activity can be detected in the erythroblasts of erythroleukemia and focally in lymphoblasts of ALL. α-Naphthyl butyrate exhibits strong activity in the monocytic cells (e.g., monoblasts, promonocytes, monocytes) but very weak or absent activity in granulocytes, megakaryocytes, and lymphocytes. The combined method for α-naphthyl butyrate esterase and chloroacetate esterase provides an objective means for demonstrating monocytes and granulocytes simultaneously in cytologic preparations and, thus, is useful for differentiating myeloblasts from monoblasts.[84,85]

α-Naphthyl esterase is useful in diagnosing acute myelomonocytic and acute monocytic leukemias (Table 37-12; Web Table 37-11).[86] To differentiate monocytes from other cells that occasionally show positivity, sodium fluoride can be added to the



■ **FIGURE 37-23** Blasts of acute monoblastic leukemia showing a positive nonspecific esterase (NSE) stain (black granulation) (bone marrow; NSE stain; 1000× magnification).

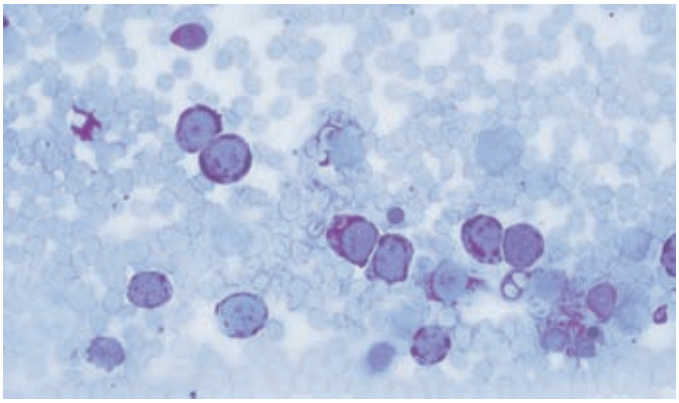
incubation mixture to inactivate the monocytic enzyme. This is referred to as the *fluoride inhibition test* in which two incubation mixtures are prepared, one without fluoride and the other with it. Slides are incubated in the incubation mixtures, and the staining patterns of the two slides are compared. The slide stained in the incubation mixture containing fluoride shows an inhibition of staining in monocytes but not in other cell lines.

Periodic Acid-Schiff

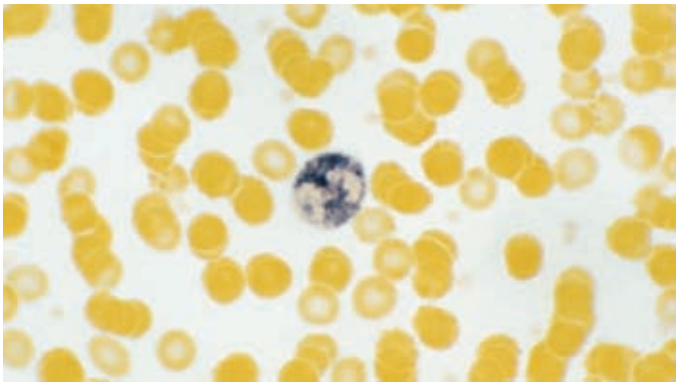
Periodic acid oxidizes 1,2-glycol groups to dialdehydes. These aldehyde groups combine with Schiff's reagent to produce a magenta product (Figure 37-24 ■). The stainable compound in the blood cells is primarily glycogen. Cellular components containing polysaccharides, mucopolysaccharides, and glycoproteins possess the 1,2-glycol groups and will be stained.[87] Mature granulocytes (the intensity of staining increases with maturity), platelets, megakaryocytes, and monocytes are stained with periodic acid-Schiff (PAS). However, the early myeloid cells, the erythroid cells, and many of the lymphoid cells are negative with PAS. Therefore, PAS positivity in these cells can indicate abnormal glycogen metabolism and can be diagnostically important. The use of this stain in diagnosing acute leukemias is summarized in Web Table 37-11.

Leukocyte Alkaline Phosphatase

The determination of **leukocyte alkaline phosphatase (LAP)** activity can be used to differentiate chronic myelogenous leukemia from leukemoid reaction/reactive neutrophilia.[88,89] In this procedure freshly prepared peripheral blood smears obtained from finger stick capillary blood or samples anticoagulated with heparin are dried for at least 1 hour prior to fixation in 60% citrate buffered acetone.[88] Fixed slides can be stored overnight in the freezer. The smears are incubated in naphthol AS-MX phosphate at an alkaline pH. The liberated naphthol immediately couples with a diazonium salt (e.g., fast blue RR), forming brown to black particles in the cytoplasm of the cells at the enzyme sites (Figure 37-25 ■). After counterstaining, the smears are evaluated microscopically. Because this enzyme is found within the secondary granules of maturing granulocytes, 100 segmented neutrophils/bands are counted, and each cell is graded using a scale of 0–4+ according to the appearance and intensity of the precipitated dye (Table 37-13 ★). The number of cells counted in each grade is multiplied by that grade, and the products are summed to obtain a total LAP score (Table 37-14 ★). Improperly stored smears and those prepared from blood anticoagulated with EDTA can give falsely low LAP scores. The range of normal scores is 13–130, although this could vary slightly in each laboratory. The range of possible values is 0–400. A score higher than 160 is generally considered increased and lower than 13 is considered decreased. The LAP scores can be increased in leukemoid reaction (infection, inflammation), polycythemia vera, pregnancy, newborns, stress, oral contraceptives, and medications (steroids, estrogen, lithium, growth factors). In secondary erythrocytosis, idiopathic myelofibrosis, and essential thrombocytosis, the enzyme activity is usually normal. LAP scores can be decreased in chronic myelocytic leukemia (CML), paroxysmal nocturnal hemoglobinuria, immune thrombocytopenia and sometimes myelodysplasia.



■ **FIGURE 37-24** PAS stain showing positive reaction (red-purple cytoplasm) of erythroblasts in acute erythroid leukemia (bone marrow; PAS stain; 400× magnification).



■ **FIGURE 37-25** LAP stain showing neutrophil with a score of 3+. Note strong dark colored granulation (peripheral blood; LAP stain; 1000× magnification).

★ **TABLE 37-13** Leukocyte Alkaline Phosphatase: Cell Rating and Staining Characteristics

Cell Rating	Amount	Intensity of Staining
0	None	None
1+	<50%	Faint
2+	50–75%	Moderate
3+	75–100%	Strong
4+	100%	Intense

The LAP score for CML patients in blast crisis or with concurrent infections can be increased.

☑ CHECKPOINT 37-16

A pathology resident is evaluating the peripheral blood smear of a 40-year-old female. The resident suspects CML. To confirm this suspicion, the resident ordered an LAP score. Do you think that the LAP score will be helpful in making the correct diagnosis?

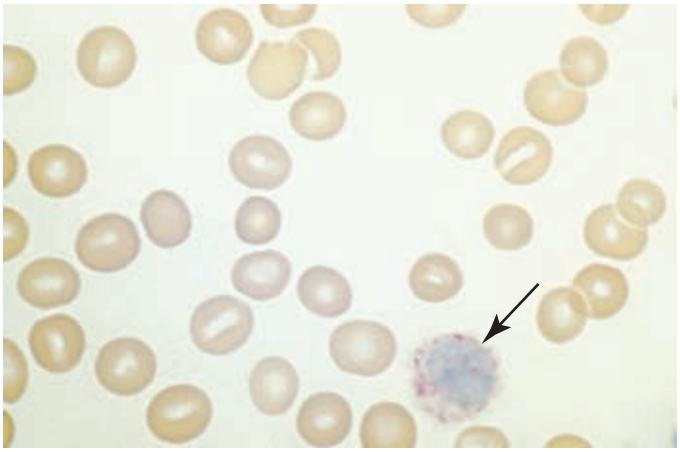
Acid Phosphatase and Tartrate-Resistant Acid Phosphatase (TRAP)

Acid phosphatase activity is detected by incubating the sample in naphthol AS-BI phosphoric acid.[90–94] Enzyme activity liberates naphthol AS-BI. The liberated naphthol immediately couples with a diazonium salt (e.g., fast garnet), forming an insoluble, visible pigment at the site of enzyme activity. Acid phosphatase activity is present in most normal leukocytes appearing as purplish, dark red intracellular granules distributed throughout the cytoplasm. A focal polarized acid phosphatase activity pattern is characteristically seen in T-cell ALL.

Seven acid phosphatase isoenzymes (0, 1, 2, 3, 3b, 4, and 5) are present in leukocytes. These isoenzymes are cell specific with neutrophils containing isoenzymes 2 and 4, lymphocytes and platelets isoenzyme 3, blasts isoenzyme 3b, and hairy cells isoenzyme 5. All but isoenzyme 5 are sensitive to tartrate inhibition. The hairy cells of hairy cell leukemia exhibit acid phosphatase activity but can be differentiated from other leukocytes using tartrate inhibition.[93,94] Tartrate inhibits the acid phosphatase activity within normal leukocytes, whereas enzyme activity within hairy cells is resistant to tartrate

★ **TABLE 37-14** Example of a LAP Score Calculation

Cell Rating	Number of Cells Counted	LAP Score
0	45	0
1+	30	30
2+	15	30
3+	5	15
4+	5	20
		Total LAP score 95



■ **FIGURE 37-26** TRAP stain showing a positive reaction of hairy cell (arrow) with acid phosphatase that is resistant to tartrate inhibition (reddish granulation) (peripheral blood; TRAP stain; 1000× magnification).

inhibition (Figure 37-26 ■). Therefore, hairy cell leukemia is said to be strongly **tartrate-resistant acid phosphatase (TRAP)** positive. Weak to moderate TRAP positivity occurs in activated lymphocytes and Sézary cells, a few cases of chronic lymphocytic leukemia, and prolymphocytic leukemia. Mast cells are also TRAP positive, and the morphology of abnormal mast cells on tissue sections is similar to that of hairy cell leukemia. Therefore, toluidine blue stain is helpful in distinguishing mast cells (positive) from hairy cells (negative).

☑ CHECKPOINT 37-17

A 30-year-old male presented to the emergency room complaining of fatigue, weakness, and gum bleeding. The CBC showed anemia, thrombocytopenia, and a WBC of 30 × 10^3/mcL. The peripheral blood smear revealed numerous intermediate to large blasts with fine nuclear chromatin and abundant cytoplasm. The bone marrow biopsy contained 60% blasts. Careful inspection detected no Auer rods. Several cytochemical stains were then performed. The blasts failed to stain with myeloperoxidase, SBB, and specific esterase; however, the majority of blasts reacted intensely with α-naphthyl esterase. Incubation with sodium fluoride inhibited the staining seen with α-naphthyl esterase. What type of leukemia do you think this patient has?

Terminal Deoxynucleotidyl Transferase

Terminal deoxynucleotidyl transferase (TdT) is an intranuclear DNA polymerase responsible for the template-independent addition of deoxyribonucleotides to the 3′-hydroxyl terminus of oligonucleotide primers during immunoglobulin and T-cell receptor gene rearrangements. TdT is normally present in thymus lymphocytes, pre-B

lymphocytes (hematogones), and 1–3% of normal bone marrow cells (blasts). It is not normally present in peripheral blood lymphocytes or lymph node lymphocytes. Because TdT is primarily found in lymphoid precursors, its detection is useful in distinguishing ALL from malignant lymphoma.[95,96] However, a positive TdT result in an acute leukemia should be interpreted with caution because TdT has been observed in up to 20% of AMLs and specifically in >90% of AML-minimally differentiated cases. The detection of TdT-positive cells within a sample is most often determined by immunophenotyping with flow cytometry and fluorescent-tagged monoclonal antibodies directed against TdT. The indirect immunofluorescence technique is another method used to detect TdT-positive cells. This method can be advantageous in diagnosing certain neoplasms as the TdT-positive cells are observed within the context of the tissue's architecture (i.e., bone marrow or lymph node biopsy).

In the indirect immunofluorescence technique for detection of TdT, rabbit anti-calf TdT is applied to the sample (e.g., bone marrow aspirate smear).[97] If the cells' nuclei contain TdT, this antibody binds to it, creating an antibody–antigen complex. Excess anti-calf TdT is washed away. A secondary antibody, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antiserum, is used to identify the antibody–antigen complex. The nucleus of cells containing TdT fluoresce.

☑ CHECKPOINT 37-18

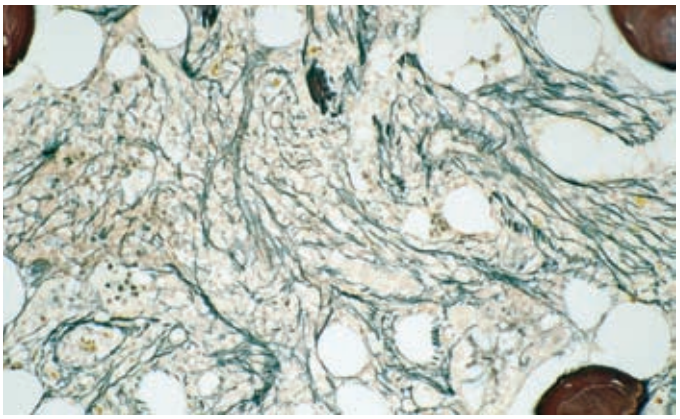
A pathologist is reviewing the slides from the pleural fluid of a 50-year-old male who has hepatosplenomegaly and minimal lymphadenopathy. It is not clear whether the mononuclear cells are mature lymphoma cells or leukemic blasts. There is not enough material for flow cytometry to do immunophenotyping, but there is enough to make a few extra cytospin smears. Which stain do you think would be useful in this case?

Toluidine Blue

Toluidine blue is a basic dye that reacts with acid mucopolysaccharides to form red to purple metachromatic granules. Bone marrow trephine sections are stained with 0.1% toluidine blue in ethanol and then examined microscopically. A positive reaction is specific for basophils and mast cells. This stain is useful in diagnosing mast cell disease and rare cases of AML with basophilic differentiation.[85,98,99] Because acid mucopolysaccharides can be decreased or absent in neoplastic disorders, a negative reaction does not rule out a neoplasm of these cells.

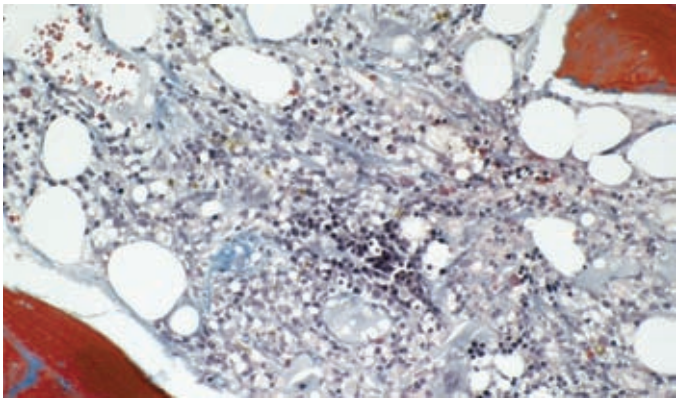
Reticulin Stain and Masson's Trichrome Stain

The reticulin and trichrome stains are used to evaluate the presence and extent of fibrosis within the bone marrow. In the Gomori methenamine silver staining method, deparaffinized bone marrow trephine sections are incubated in the following solutions: 0.5% periodic acid, methenamine silver working solution (contains methenamine, silver nitrate, and sodium borate), 0.2% gold chloride, and 3% sodium thiosulfate. The section is counterstained with nuclear fast red and examined microscopically. The reticulin fibers appear black. This silver staining method is used to examine reticulin fibers that form the framework of the bone marrow. In a hypercellular marrow, the reticulin becomes more prominent, but its structural framework is preserved. Reticulin in myelofibrosis is markedly increased and the structural framework is distorted (Figure 37-27 ■).



■ **FIGURE 37-27** Bone marrow biopsy stained with reticulin stain showing fibrosis (black fibers) in a patient with myelofibrosis (bone marrow; paraffin section, reticulin stain; 200× magnification).

In the Masson's trichrome stain, deparaffinized bone marrow trephine sections or frozen trephine sections are stained with Weigert's iron hematoxylin working solution and then incubated in the following solutions: Biebrich scarlet-acid fuchsin, phosphomolybdic acid, and aniline blue. The slides are examined microscopically; collagen fibers appear blue, nuclei black, and the background red. The trichrome stain is used to evaluate collagenous fibrosis within the bone marrow. Normally, collagenous fibrosis is focal and perivascular. Increased staining can be observed in myelofibrosis and indicates significant fibrosis (Figure 37-28 ■).



■ **FIGURE 37-28** Bone marrow biopsy stained with Masson's trichrome stain showing collagenous fibrosis (fibers are stained light blue) in a patient with myelofibrosis (bone marrow; paraffin section, Masson's Trichrome stain, 400× magnification)

Summary

This chapter reviewed the routine and reflex tests performed within the hematology laboratory. The accuracy and reliability of the results depend on the laboratory professional's knowledge of each procedure, which is necessary to understand the appropriate use, troubleshoot potential sources of error, identify other problems in performing the test, and understand the meaning of test results. Although the majority of the routine hematology tests are performed by automated instrumentation, the basic principles, applications, and potential sources of error hold true for the automated adaptation of each procedure. The results obtained from the routine and reflex test procedures are utilized in diagnosing, prognosing, and therapeutic monitoring a variety of disorders including anemias, leukemias, infections, and inherited leukocyte disorders.

Review Questions

Level I

1. What is the appropriate sequence to fill sample collection tubes according to the color of each top? (Objective 6)
   A. red, lavender, blue, green
   B. lavender, blue, red, green
   C. blue, red, green, lavender
   D. red, blue, lavender, green
2. The function of a microscope's condenser is to: (Objective 8)
   A. magnify the light beam before striking the sample
   B. collect the diffracted light from the sample
   C. direct the light beam onto the sample
   D. project diffracted light to the objective
3. A manual leukocyte count was performed on an EDTA-anticoagulated sample. The sample was diluted 1/20, and a total of 165 leukocytes were counted in the four corner squares of the hemacytometer. What is the leukocyte count? (Objective 19)
   A. 1.3×10^3/mcL
   B. 3.3×10^3/mcL
   C. 4.1×10^3/mcL
   D. 8.3×10^3/mcL
4. Any turbidity in a peripheral blood sample results in a falsely elevated hemoglobin determination. Which of the following is a potential source of turbidity? (Objective 19)
   A. lipemia
   B. leukocyte count $= 18.0 \times 10^9$/L
   C. increased levels of carboxyhemoglobin
   D. presence of hemoglobin F
5. Which of the following is *not* a condition associated with an elevated ESR? (Objective 19)
   A. rheumatoid arthritis
   B. polycythemia vera
   C. multiple myeloma
   D. osteomyelitis
6. Which of the following is observed if a purple top collection tube is underfilled? (Objective 6)
   A. falsely elevated erythrocyte count
   B. falsely decreased microhematocrit
   C. falsely elevated platelet count
   D. falsely decreased hemoglobin
7. In performing a reticulocyte count, the laboratory professional observes suspicious light bluish-green bodies at the periphery of some erythrocytes. What is the appropriate course of action? (Objective 19)
   A. These bodies are aggregated reticulum, and erythrocytes containing them should be tabulated as reticulocytes.
   B. These bodies contain iron and should be confirmed using the Prussian blue stain.
   C. These bodies are aggregated DNA, and erythrocytes containing them should not be tabulated as reticulocytes.
   D. These bodies are denatured hemoglobin, and erythrocytes containing them should not be tabulated as reticulocytes.

8. The laboratory is experiencing problems with the air conditioning system and is unusually warm. The effect of this temperature change on the ESRs performed during this time period is that they: (Objective 19)

   A. will be falsely elevated because higher temperature promotes sedimentation

   B. will be falsely decreased because erythrocytes will have a higher ζ potential

   C. will be unaffected because erythrocyte sedimentation does not depend on temperature

   D. will be falsely elevated because erythrocytes will become swollen

9. When examining an acid elution test for hemoglobin F, the laboratory professional observed uniformly stained erythrocytes on the control slide (mixture of adult erythrocytes and cord cells) and on the patient's slide. What is the source of this error? (Objective 19)

   A. fixative

   B. stain

   C. elution buffer

   D. counterstain

10. Alkaline hemoglobin electrophoresis reveals a band of hemoglobin with HbC mobility. Citrate agar electrophoresis does *not* show an HbC band. The most likely explanation is that the: (Objective 19)

    A. abnormal hemoglobin can be HbE or HbO_{Arab}

    B. citrate agar electrophoresis result is erroneous

    C. abnormal hemoglobin can be HbG or HbD

    D. alkaline hemoglobin electrophoresis is not reliable

Level II

1. A laboratory professional experiences difficulty identifying platelets using phase microscopy. What should be done to improve this? (Objective 2)

   A. lower the condenser to increase the depth of field

   B. align the annulus with the phase-shifting element

   C. perform Koehler illumination with the 100× objective

   D. decrease the brightness dial to increase resolution

2. An EDTA-anticoagulated blood sample was diluted 1/100 for a manual platelet count. A total of 526 platelets were counted in the center square on one side of the hemacytometer. Which of the following clinical conditions is associated with this result? (Objective 7)

   A. immune thrombocytopenia

   B. megaloblastic anemia

   C. acute leukemia

   D. iron-deficiency anemia

3. If the solubility test for hemoglobin S is positive, the appropriate reflex test is: (Objective 6)

   A. alkaline hemoglobin electrophoresis

   B. hemoglobin A_2 by column chromatography

   C. isoelectric focusing with polyacrylamide gel

   D. hemoglobin F determination by acid elution

4. While evaluating the erythrocyte morphology on a Wright-stained peripheral blood smear, the laboratory professional observed many crenated erythrocytes. What corrective action will minimize the presence of these cells? (Objective 4)

   A. Allow the sample to stabilize for at least 4 hours and then prepare the blood smear.

   B. Dry the blood smear quickly after its preparation.

   C. Increase the spreader speed to prepare the blood smear.

   D. No corrective action is available.

5. A laboratory professional noted that a patient's platelet count was significantly decreased. In examining the patient's blood smear, platelets appeared to adhere to the neutrophils. How would you obtain an accurate platelet count? (Objective 4)

   A. Recollect the sample in citrate and reanalyze the platelet count.

   B. Allow the sample to set at 25°C for 5 hours and reanalyze the platelet count.

   C. Warm the sample at 37°C for 15 minutes and reanalyze the platelet count.

   D. No corrective action is available.

6. Which screening test can be used if a diagnosis of PCH is suspected? (Objective 6)

   A. alkaline hemoglobin electrophoresis

   B. Donath-Landsteiner test

   C. Coomb's denaturation test

   D. acid elution test

7. A patient had the following results:

RBC	2.90×10^6/mcL	MCV	76 fL
Hb	6.2 g/dL	MCH	21 pg
Hct	22.0%	MCHC	28 g/dL
Serum iron	45 mcg/dL		
sTfR	28.5 nmol/L		

   With what type of anemia are these results associated? (Objective 7)

   A. iron-deficiency anemia

   B. β thalassemia minor

   C. anemia of chronic disease

   D. sideroblastic anemia

8. In performing a reticulocyte count, the laboratory professional observes erythrocyte inclusions suggesting denatured hemoglobin. What is the best stain to confirm the identity of these inclusions? (Objective 6)

   A. crystal violet stain

   B. Wright's stain

   C. brilliant green stain

   D. methyl violet stain

9. You are performing several cytochemical stains on a bone marrow sample from a patient recently diagnosed with acute leukemia. You are examining the MPO slide, and the blasts are negative. However, the reagent is nearing its expiration date, and you question whether the stain worked properly. Which cells found in the bone marrow normally express myeloperoxidase and could be used to assess the stain's integrity? (Objective 6)

   A. neutrophils

   B. red cell precursors

   C. megakaryocytes

   D. lymphocytes

10. A patient's CBC and peripheral blood smear examination suggests a diagnosis of CML. The physician orders an LAP score to be performed on the current hematology sample (lavender top tube). What should the laboratory professional tell the physician? (Objective 6)

    A. The current hematology sample is acceptable, and the procedure will be completed today.

    B. The current hematology sample is unacceptable because it would result in a falsely decreased LAP score. A new sample must be collected using heparin.

    C. The current hematology sample is unacceptable because it would result in a falsely elevated LAP score. A blood smear should be obtained by capillary puncture.

    D. The current hematology sample is acceptable; however, the LAP score needs to be corrected for the dilutional effect of the anticoagulant.

Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

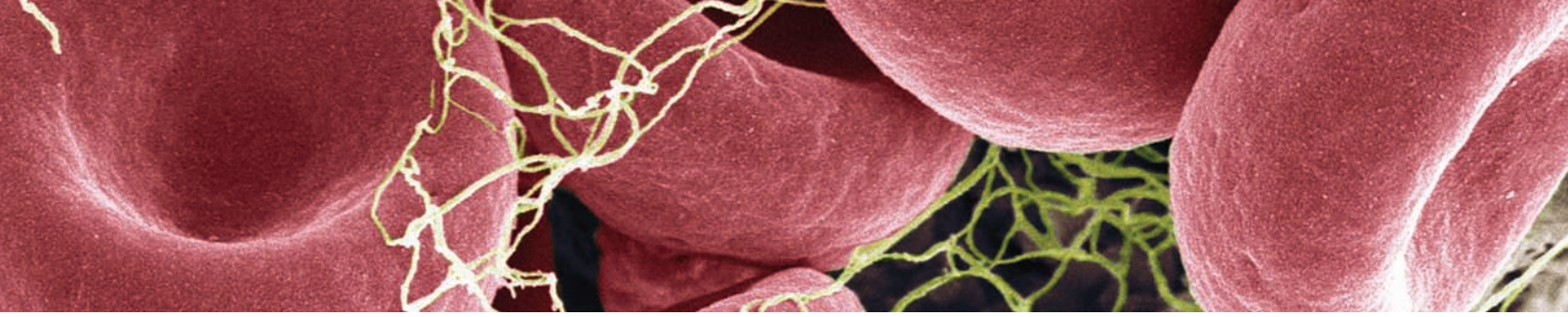
The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Lewandrowski K, Gregory K, Macmillan D. Assuring quality in point-of-care testing: evolution of technologies ,informatics, and program management. *Arch Pathol Lab Med*. 2011;135:1405-14.
2. Clinical and Laboratory Standards Institute. *Tubes and Additives for Venous and Capillary Blood Specimen Collection. Approved Standard*, 6th ed. H01-A6. Wayne, PA: CLSI; 2010.
3. Clinical and Laboratory Standards Institute. *Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays. Approved Guideline*, 5th ed. H21-A5. Wayne, PA: CLSI; 2008.
4. Clinical and Laboratory Standards Institute. *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture: Approved Standard*, 6th ed. H3-A6. Wayne, PA: CLSI; 2007.
5. Joint Commission Perspectives. *2008 National Patient Safety Goals*. 2007;27(7). Washington DC: The Joint Commission.
6. Clinical and Laboratory Standards Institute. *Procedures and Devices for the Collection of Diagnostic Capillary Blood Specimens: Approved Standard*, 6th ed. H04-A6. Wayne, PA: CLSI; 2008.
7. Occupational Safety and Health Administration. *Occupational Exposure to Bloodborne Pathogens Standard*. 29 CFR 1910.1030. U.S. Government, Washington, DC. 1992.
8. Occupational Safety and Health Administration. Occupational Safety and Health Administration Instruction, CPL 02-02-069. *Enforcement Procedures for the Occupational Exposure to Bloodborne Pathogens*. 29 CFR 1910.1030. U.S. Government, Washington, DC, 2001.
9. Occupational Safety and Health Administration. *OSHA Revised Bloodborne Pathogens Standard*, April 18, 2001. 29 CFR 1910.1030. U.S. Government, Washington, DC. 2001.
10. Nikon, Inc. *Instructions for Labophot-2*. Garden City, NY: Nikon; 1990.
11. Nikon, Inc. *Introduction to the Microscope: Operation and Preventive Maintenance Using the Nikon Labophot*. Garden City, NY: Nikon; 1981.
12. Murphy DB. *Fundamentals of Light Microscopy and Electronic Imaging*. New York: Wiley-Liss; 2001.
13. Nikon, Inc. *CF160 Optics Overview*. Garden City, NY: Nikon; 2000.
14. Keller E. Light microscopy and cell structure. In DL Spector, RD Goldman, LA Leinwand, eds. *Cells: A Laboratory Manual*. Vol. 2. Woodbury, NY : Cold Spring Harbor Laboratory Press; 1997.
15. Clinical and Laboratory Standards Institute. *Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods: Approved Standard*, 2nd ed. Wayne, PA: CLSI; 2007.
16. Steine-Martin EA. Causes of poor leukocyte distribution in manual spreader slide blood films. *Am J Med Tech*. 1980;46:624–32.
17. Benattar L, Flandrin G. Comparison of the classical manual pushed wedge films with an improved automated method for making blood smears. *Hematol Cell Ther*. 1999;41:211–15.
18. Ryan DH. Examination of blood cells. In: Kaushansky K, Lichtman MA, Beutler E, et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010.
19. National Committee for Clinical Laboratory Standards. *Romanowsky Blood Stains*. Villanova, PA: NCCLS; 1986.
20. Marshall PN. Romanowsky staining: state of the art and ideal techniques. In: JA Koepke, ed. *Differential Leukocyte Counting*. Skokie, IL: College of American Pathologists; 1979.
21. Kopecke JA. Standardization of the manual differential leukocyte count. *Lab Med*. 1980;11:371–75.

22. Koepke JA, Dotson MA, Shifman MA. A critical evaluation of manual/visual differential leukocyte counting method. *Blood Cells*. 1985;11:173.
23. Gulati GL, Hyun BH. Blood smear examination. *Hematol Oncol Clin North Am*. 1994;8(4):631–50.
24. *Leuko-TIC® 1:20 • Blue Single Tests for Quick, Simple, Clean and Precise Counting of WBC*. Umkirch/Freiburg, Germany: bioanalytic GmbH; 2011 (www.bioanalytic.de).
25. *Ery-TIC® 1:200 Single tests for quick, simple, clean, and precise counting of RBC*. Umkirch/Freiburg, Germany: bioanalytic GmbH: 2010. (www .bioanalytic.de).
26. *Thrombo-TIC® 1:100 Single Tests for Quick, Simple, Clean, and Precise Counting of Platelets (PLT)*. Umkirch/Freiburg, Germany: bioanalytic GmbH: 2011. www.bioanalytic.de).
27. Clinical and Laboratory Standards Institute. *Reference and Selected Procedures for the Quantitative Determination of Hemoglobin in Blood*, 3rd ed. H15-A3. Wayne, PA: CLSI; 2000.
28. International Committee for Standardization in Haematology; Expert Panel on Haemoglobinometry. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1986) and specifications for international haemiglobincyanide reference preparation, 3rd ed. *Clin Lab Haematol*. 1987;9(1):73–79.
29. Clinical and Laboratory Standards Institute. *Procedure for Determining Packed Cell Volume by the Microhematocrit Method*, 3rd ed. H7-A3. Wayne, PA: CLSI; 2000.
30. International Committee for Standardization in Hematology. Selected methods for the determination of packed cell volume. In: van Assendelft OW, England JM, eds. *Advances in Hematologic Methods: The Blood Count*. Boca Raton, FL: CRC Press; 1982.
31. Clinical and Laboratory Standards Institute. *Procedures for the Erythrocyte Sedimentation Rate Test; Approved Standard*, 5th ed. H02-A5. Wayne, PA: CLSI; 2011.
32. International Council for Standardization in Hematology. ICSH review of measurement of the erythrocyte sedimentation rate. *Int J Lab Hem*. 2011;33:125–32.
33. Rabjohn L, Roberts K, Troiano M et al. Diagnostic and prognostic value of erythrocyte sedimentation rate in contiguous osteomyelitis of the foot and ankle. *J Foot Ankle Surg*. 2007;46(4):230.
34. Andresdottir MB, Sigfusson N, Sigvaldason H et al. Erythrocyte sedimentation rate, an independent predictor of coronary heart disease in men and women, the Reykjavik study. *Am J Epidemiol*. 2003;158(9):844–51.
35. Nayak AR, Kashyap RS, Kabra D et al. Evaluation of routinely performed hematological and biochemical parameters for the prognosis of acute ischemic stroke patients. *Neurol Sci*. 2011;32:855–60.
36. Giavarina D, Dall'Olio G, Soffiati G. Method comparison of automated systems for the erythrocyte sedimentation rate. *Am J Clin Pathol*. 1999;112(5):721–2, author reply 723–4.
37. Cerutti H, Muzzi C, Leoncini R et al. Erythrocyte sedimentation rate measurement by VES Matic Cube 80 in relation to inflammation plasma proteins. *J Clin Lab Anal*. 2011;25:198–202.
38. Curvers J, Kooren J, Laan M et al. Evaluation of the Ves-Matic Cube 200 erythrocyte sedimentation method: comparison with Westergren-based methods. *Am J Clin Pathol*. 2010;134:653–60.
39. Perovic E, Bakovic L, Valcic A. Evaluation of the Ves-Matic Cube 200—an automated system for the measurement of the erythrocyte sedimentation rate. *Int J Lab Hem*. 2010;32:88–94.
40. *Streck ESR-auto plus® and ESR-657 mixer operator's manual (Part 320578-2)*. Omaha, NE: Streck; 2011.
41. Vennapusa B, De La Cruz L, Shah H et al. Erythrocyte sedimentation rate (ESR) measured by the Streck ESR-Auto Plus is higher than with the Sediplast Westergren Method—a validation study. *Am J Clin Pathol*. 2011;135:386–90.
42. Gambino R. C-reactive protein—undervalued, underutilized. *Clin Chem*. 1997;43(11):2017–18.
43. Young B, Gleeson M, Cripps AW. C-reactive protein: a critical review. *Pathology*. 1991;23(2):118–24.
44. Torres JL, Ridker PM. Clinical use of high sensitivity C-reactive protein for the prediction of adverse cardiovascular events. *Curr Opin Cardiol*. 2003;18:471–8.
45. Roberts WL. CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice: laboratory tests available to assess inflammation—performance and standardization: a background paper. *Circ*. 2004;110:e572–6.
46. Molino-Lova R, Macchi C, Gori AM et al. High sensitivity C-reactive protein predicts the development of new carotid artery plaques in older persons. *Nutr Metab Cardiovasc Dis*. 2011;21:776–82.
47. Firooz N, Albert DA, Wallace DJ et al. High-sensitivity C-reactive protein and erythrocyte sedimentation rate in systemic lupus erythematosus. *Lupus*. 2011;20:588–97.
48. Aletaha D, Neogi T, Silman AJ et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative intiative. *Arthritis Rheu*. 2010;62(9):2569–81.
49. Funovits J, Aletaha D, Bykerk V et al. The 2010 American College of Rheumatology/European League against Rheumatism classification criteria for rheumatoid arthritis: methodical report phase 1. *Ann Rheum Dis*. 2010;69:1589–95.
50. Neogi T, Aletaha D, Silman AJ et al. The 2010 American college of rheumatology/European league against rheumatism classification criteria for rheumatoid arthritis: phase 2 methodological report. *Arthritis Rheum*. 2010;62(9):2582–91.
51. Miyachi H, Asai S, Takemura Y. A cost-effectiveness evaluation of reticulocyte measurement in new outpatients with or without hematologic disorders. *Clin Chem Lab Med*. 2006;44(8):1035–8.
52. Wiwanitkit V. Comparison of cost effectiveness between measuring the serum erythropoietin level and reticulocyte count for monitoring thalassemic patients: a note in thai beta thalassemia/Hb E subjects. *Hematology*. 2004:9(4):311–3.
53. Serke S, Riess H, Oettle H et al. Elevated reticulocyte count: a clue to the diagnosis of haemolytic-uraemic syndrome (HUS) associated with gemcitabine therapy for metastatic duodenal papillary carcinoma: a case report. *Br J Cancer*. 1999;79:1519–21.
54. Bhandari S, Norfolk D, Brownjohn A et al. Evaluation of RBC ferritin and reticulocyte measurements in monitoring response to intravenous iron therapy. *Am J Kid Dis*. 1997;30(6):814–21.
55. Clinical and Laboratory Standards Institute. *Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes): Approved Standard*, 2nd ed. H44-A2. Wayne, PA: CLSI; 2004.
56. National Committee for Clinical Laboratory Standards. *Solubility Test to Confirm the Presence of Sickling Hemoglobins*, 2nd ed. H10-A2. Villanova, PA: NCCLS; 1995.
57. Natarajan K, Townes TM, Kuttar A . Disorders of hemoglobin structure: sickle cell anemia and related abnormalities. In: Kaushansky K, Lichtman M, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill Medical; 2010.
58. National Committee for Clinical Laboratory Standards. *Detection of Abnormal Hemoglobin Using Cellulose Acetate Electrophoresis*, 2nd ed. Wayne, PA: NCCLS; 1994.
59. *SPIFE Alkaline Hemoglobin Electrophoresis Procedure*. Beaumont, TX: Helena Laboratories; 2004.
60. *SPIFE Acid Hemoglobin Procedure*. Beaumont, TX: Helena Laboratories; 2006.
61. *Beta-Thal HbA_2 Quik Column Procedure*. Beaumont, TX: Helena Laboratories; 2004.
62. Kalleas C, Tentes I, Margaritis D et al. Effect of Hb S in the determination of HbA_2 with the Biorad Variant II analyzer. *Clin Biochem*. 2007;40:744–46.
63. Van Kirk R, Sandhaus LM, Hoyer JD. The detection and diagnosis of hemoglobin A_2 by high-performance liquid chromatography. *Am J Clin Pathol*. 2005;123:657–61.
64. Riou J, Godart C, Mathis M et al. Evaluation of the Bio-Rad VARIANT™ II HbA_2/HbA1c Dual Program for measurement of hemoglobin concentrations and detection of variants. *Clin Chem Lab Med*. 2005;43:237–43.
65. Keren DF, Hedstrom D, Gulbranson R et al. Comparison of Sebia Capillarys capillary electrophoresis with the Primus high-pressure liquid chromatography in the evaluation of hemoglobinopathies. *Am J Clin Pathol*. 2008;130:824–31.
66. Mais DD, Gulbranson R, Keren DF. The range of hemoglobin A2 in hemoglobin E heterozygotes as determined by capillary electrophoresis. *Am J Clin Pathol*. 2009;132:34–38.
67. *Fetal Hemoglobin-Acid Elution: Semi-Quantitative Procedure for Blood Smears*. St. Louis: Sigma-Aldrich Diagnostics; 2005.
68. National Committee for Clinical Laboratory Standards. *Quantitative Measurement of Fetal Hemoglobin Using the Alkali Denaturation Method*. Wayne, PA: NCCLS; 1989.
69. Fibach E. Flow cytometric analysis of fetal hemoglobin in erythroid precursors of β-thalassemia. *Clin Lab Haemtol*. 2004;26:187–93.

70. Huisman THJ, Jonxis JHP. *The Hemoglobinopathies: Techniques of Identification*. New York: Marcel Decker; 1977.
71. Kutler F. Diagnostic approach to hemoglobinopathies. *Hemoglobin*. 2007;31(2):243–50.
72. Schwab ML, Lewis AE. An improved stain for Heinz bodies. *Am J Clin Pathol. 1969;51(5):673.*
73. *UNOPETTE RBC Osmotic Fragility Determination for Manual Methods Procedure*. Rutherford, NJ: Becton, Dickinson; 1996.
74. Gallagher PG. The red blood cell membrane and its disorders: hereditary spherocytosis, elliptocytosis, and related diseases. In: Kaushansky K, Lichtman M, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill Medical; 2010:617–46.
75. AABB. *American Association of Blood Banks: Technical Manual*, 15th ed. Bethesda, MD: AABB; 2005.
76. *Quantikine IVD Human Epo Immunoassay Procedure*. Minneapolis, MN: R&D Systems; 2011.
77. Prchal JT. Primary and secondary polycythemias (erythrocytosis). In: Kaushansky K, Lichtman M, Beutler E et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill Medical; 2010:823–38.
78. *Quantikine IVD Human sTfR Immunoassay Procedure*. Minneapolis, MN: R&D Systems; 2010.
79. Sigma-Aldrich, Inc. *Peroxidase (Myeloperoxidase)*. St. Louis: Sigma-Aldrich; 2003.
80. Gabbas AG, Li CY. Acute non-lymphocytic leukemia with eosinophilic differentiation. *Am J Hematol*. 1986;21:29–38.
81. Sigma-Aldrich, Inc. *Sudan Black B Staining System*. St. Louis: Sigma-Aldrich; 2003.
82. Stass SA, Pui CH, Melvin S et al. Sudan black B positive acute lymphoblastic leukaemia. *Br J Haematol*. 1984;57:413–21.
83. Sigma-Aldrich, Inc. *Naphthol AS-D Choloroacetate Esterase and Alpha-Naphthyl Acetate Esterase*. St. Louis: Sigma-Aldrich; 2006.
84. Shibata A, Bennett JM, Castoldi GL. Recommended methods for cytological procedures in hematology. *Clin Lab Haematol*. 1985;7:55–74.
85. Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. *Am J Clin Pathol*. 1971;55:283–90.
86. Li CY. Leukemia cytochemistry. *Mayo Clin Proc*. 1981;56:712–13.
87. Sigma-Aldrich, Inc. *Periodic Acid-Schiff (PAS) Staining System*. St. Louis: Sigma-Aldrich; 2003.
88. Sigma-Aldrich, Inc. *Alkaline Phosphatase*. St. Louis: Sigma-Aldrich; 2003.
89. National Committee for Clinical Laboratory Standards. *Proposed Standard: Histochemical Method for Leukocyte Alkaline Phosphatase*. Villanova, PA: NCCLS; 1984.
90. Sigma-Aldrich, Inc. *Acid Phosphatase, Leukocyte*. St. Louis: Sigma-Aldrich; 2005.
91. Li CY, Yam LT, Lam KW. Acid phosphatase isoenzyme in human leukocytes in normal and pathologic conditions. *J Histochem Cytochem*. 1970;18:473–81.
92. Li CY, Yam LT, Lam KW. Studies of acid phosphatase isoenzymes in human lymphocytes: demonstration of isoenzyme cell-specificity. *J Histochem Cytochem*. 1970;18:901–10.
93. Yam LT, Li CY, Lam KW. Tartrate-resistant acid phosphatase isoenzyme in the reticulum cells of leukemic reticuloendotheliosis. *N Engl J Med*. 1971;284:357–60.
94. Yam LT, Janckila AJ, Li CY et al. Cytochemistry of tartrate-resistant acid phosphatase: fifteen years' experience. *Leukemia*. 1987;1:285–88.
95. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. *Blood*. 1979;54:1203–15.
96. Liu L, McGavran L, Lovell MA et. al. Nonpositive terminal deoxynucleotidyl transferase in pediatric precursor B-lymphoblastic leukemia. *Am J Clin Pathol*. 2004;121:810.
97. Sun T, Li CY, Yam LT. *Atlas of Cytochemistry and Immunochemistry of Hematologic Neoplasms*. Chicago: American Society of Clinical Pathologists Press; 1985.
98. Li CY, Yam LT. Cytochemical characterization of leukemic cells with numerous cytoplasmic granules. *Mayo Clin Proc*. 1987;62:978–85.
99. Wick MR, Li CY, Pierre RV. Acute non-lymphocytic leukemia with basophilic differentiation. *Blood*. 1982;60:38–45.



38 Bone Marrow Examination

AAMIR EHSAN, MD
ANDREA YUNES MD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Identify the sites for obtaining bone marrow samples.
2. List indications for the need to perform bone marrow studies.
3. Explain the difference between core biopsy and bone marrow aspirate and the use of each.
4. Describe how to perform a bone marrow differential count.
5. Define *myeloid-to-erythroid (M:E) ratio* and explain what can cause an increase or decrease in the ratio.
6. Give the expected bone marrow cellularity for infants, adults, and older adults.
7. Describe how to estimate bone marrow cellularity and iron stores and to interpret results.
8. Explain the role of the hematology laboratory in bone marrow evaluation.
9. List the reasons that special stains are used on bone marrow specimens.

Objectives—Level II

Upon completion of this unit of study, the student should be able to:

1. Differentiate benign lymphoid aggregates and malignant lymphoma.
2. Evaluate the bone marrow specimen and select the appropriate specimens for ancillary studies.
3. Perform a bone marrow differential count and calculate and interpret the myeloid-to-erythroid (M:E) ratio.
4. Estimate and interpret bone marrow cellularity.
5. Correlate results of marrow iron stores with various diseases.
6. Describe the role of the clinical laboratory professional in the bone marrow procedure.
7. Identify key features of a bone marrow report.

Chapter Outline

Key Terms

Dry tap
Granulomatous
Hematogone
Immunohistochemical stain
Ring sideroblast

Background Basics

The information in this chapter builds on concepts from previous chapters. To maximize your learning experience, you should review these concepts before beginning this chapter:

Level I

- Review the development and differentiation of hematopoietic cells. (Chapter 4)
- Describe the structure and function of the bone marrow. (Chapter 3)
- Describe normal erythropoiesis and leukopoiesis. (Chapters 4, 5, 7–9)

Level II

- Identify the changes in hematopoiesis that lead to bone marrow hyperplasia or hypoplasia. (Chapter 3)

CASE STUDY

We will refer to this case study throughout the chapter.

Robert, a 32-year-old male, visited his primary care physician with complaints of weakness and fatigue. He had been in good health until 2 months ago when he noted he was becoming increasingly tired after usual daily work activity. Physical examination reveals no lymphadenopathy, hepatomegaly, or splenomegaly. CBC shows the following:

RBC	3.0×10^{12}/L (3.0×10^{6}/mcL)
Hb	90 g/L (9 g/L)
Hct	0.27 L/L (27%)
WBC	2.0×10^{9}/L (2.0×10^{3}/mcL)
Platelet count	30×10^{9}/L (30×10^{3}/mcL)

With these results, consider additional tests that should be done and a possible diagnosis.

OVERVIEW

Bone marrow examination can be necessary to diagnose, make a prognosis, and/or evaluate therapeutic response for a variety of hematologic and nonhematologic problems. The clinical laboratory professional is responsible for preparing the specimen and performing the preliminary examination of the bone marrow sample. This chapter discusses the indications for performing a bone marrow procedure as well as the preparation and evaluation of a bone marrow specimen. Emphasis is on processing and preparing the specimen for morphologic study. The evaluation and interpretation of bone marrow findings are discussed. Specimen requirements and indications for ancillary studies also are included. The content of the bone marrow report is described.

INTRODUCTION

Hematopoiesis begins in the yolk sac as early as 19 days gestation. At about the third month of fetal life, the liver becomes the chief site of hematopoiesis. (To a lesser extent, hematopoiesis occurs in the spleen, kidney, thymus, and lymph nodes.) In the third trimester, after birth, and throughout adult life, the bone marrow is the primary source of hematopoiesis (Chapters 3, 4).

Bone marrow, the hematopoietic compartment of bone, is a highly vascularized loose connective tissue located between the trabeculae of spongy bone. It has a volume of 30–50 mL/kg of body weight. The marrow is composed of two major compartments, hematopoietic and vascular. The hematopoietic compartment includes both hematopoietic cells and stromal cells (supporting cells). It is composed of hematopoietically active red marrow (erythroid and myeloid cells) and hematopoietically inactive yellow marrow. The yellow marrow occupies the central cavity and is composed of adipocytes. The vascular compartment of the bone marrow is composed of the nutrient artery, vein, arterioles, and sinuses (Figure 3-3).

INDICATIONS FOR BONE MARROW EVALUATION

A primary objective of a bone marrow examination is to assess the quantity and development of hematopoietic cells. Bone marrow evaluation is necessary for diagnosing, managing, making prognoses, and following up a variety of hematologic and nonhematologic disorders (Table 38-1 ★). Because the cells in the peripheral blood often reflect changes in the bone marrow, the bone marrow should always be evaluated in conjunction with the results of the peripheral blood count and smear review[1] (Chapter 10). Thrombocytopenia, coagulation factor deficiency, or anticoagulant therapy are *not* contraindications for the bone marrow procedure. However, a bone marrow biopsy should *not* be performed when a bone marrow procedure will not be useful in diagnosing or evaluating the patient's condition.

★ **TABLE 38-1** Conditions for Which a Bone Marrow Evaluation Is Indicated

Purpose	Condition
Primary diagnosis of hematopoietic and lymphoid malignancies	Acute leukemias
	Chronic myeloproliferative disorders
	Chronic lymphoproliferative disorders
	Myelodysplastic syndromes
	Hodgkin's and non-Hodgkin's lymphomas
	Plasma cell neoplasms
Staging of lymphoid malignancies and solid tumors	Lymphomas
Post-treatment follow-up	After chemotherapy and radiation therapy for neoplasms
	After stem cell transplant
Detection of infection and/or source of fever of unknown origin	Mycobacterium and fungal infections
	Granulomas
	Unknown infectious agents using cultures and special stains
	Hemophagocytic syndrome
Primary diagnosis of systemic diseases	Metabolic disorders (e.g., Gaucher's disease)
	Systemic mastocytosis
Miscellaneous	Evaluation of storage iron
	Evaluation of unexplained cytopenias

CASE STUDY *(continued from page 816)*

Review of the peripheral blood smear confirms the presence of thrombocytopenia. Red cells are normochromic and normocytic with no increase in polychromatophilic erythrocytes. A few teardrop cells are present. White blood cells show a left shift but no toxic granulation or Döhle bodies. No circulating blasts are seen.

Robert denies any history of alcohol abuse. The test for HIV is negative, and he is not on any medications. Vitamin B_{12} and folic acid levels are normal.

1. Is a bone marrow evaluation indicated in this patient? Why or why not?

BONE MARROW PROCEDURE

Sites of hematopoiesis differ by age. At birth, hematopoietic red marrow fills all bone cavities. With age, adipose tissue (fat) replaces hematopoietic tissue in some bones. By adolescence, hematopoietic marrow is only in centrally located bones including the skull, scapulae, ribs, sternum, clavicles, vertebrae, and the proximal ends of the long bones of the arms and legs. In certain disease states when there is a chronic increased demand for blood cells (e.g., thalassemia, sickle cell anemia), red marrow in the adult can extend into the long bones. Cellularity within red marrow decreases with age. At birth, red marrow is about 90% cellular and decreases to about 50% cellularity in an elderly adult. This change in sites of hematopoiesis and cellularity has implications for where to sample the bone marrow and how to interpret cellularity. Most marrow specimens are taken from the posterior superior iliac crest after adolescence (Figure 38-1 ■), although the sternum and anterior iliac crest are occasionally used in adult patients. The anteromedial surface of the tibia is sometimes used in children <2 years of age. The spines of the lumbar vertebral bodies L1 and L2 occasionally are used in older children. The composition of marrow obtained from different sites has been shown to be similar (i.e., the proportion of different cells at different sites is similar).

A bone marrow aspirate removes marrow fluid and cells from the marrow cavity of an appropriate source (see above). A bone marrow biopsy removes a plug of tissue containing bone and associated marrow, preserving (if done correctly) the three-dimensional architecture of the tissue. The bone marrow aspirates and biopsies are obtained using disposable needles that are modifications of the needle first introduced by Jamshidi.[2] A sterile technique is always used. The skin over the biopsy site is first cleaned with a disinfectant solution, and then 1–2% lidocaine (local anesthetic) is infiltrated into the skin, subcutaneous tissue, and periosteum. Some patients require additional sedation with intravenous or intramuscular medications. A small skin incision is made with a blade to facilitate needle penetration. It is recommended to perform the biopsy first and then the aspiration to avoid distorting the marrow architecture in the biopsy specimen. However, because the aspirate can clot if the biopsy is done first, some institutions prefer performing the aspirate first. If the aspiration is done first, it is recommended that the biopsy be performed at a different site on the periosteal surface.[3] In adults, a *trephine core biopsy* commonly uses an 11-gauge cutting needle to obtain a core of marrow tissue. This is followed by aspiration of about 2 mL of marrow using an 18-gauge needle.

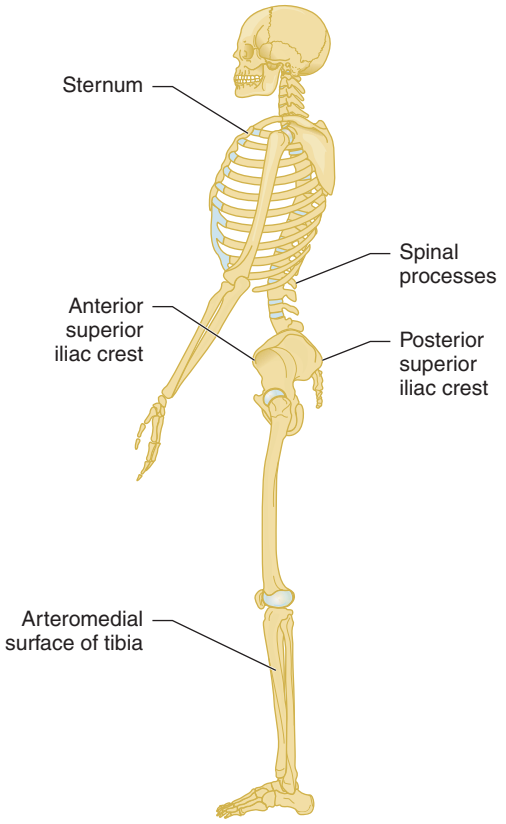


FIGURE 38-1 Sites used for aspiration and biopsy of bone marrow.

Complications of bone marrow biopsy and aspiration are rare, especially when performed by experienced practitioners. The most common complications are pain, localized bleeding, and wound infection. Most patients experience some discomfort during the aspiration procedure even with adequate infiltration of local anesthetic. If not carefully performed, it is possible to go through the entire thickness of the bone and damage the underlying tissues. This risk is higher with a sternal aspiration. After the biopsy procedure, the patient is advised to lie on the biopsy site. The site should be reevaluated in 15–30 minutes for any bleeding or oozing. In thrombocytopenic patients, applying pressure at the biopsy site and having the patients lie on their back for 20–30 minutes can control local bleeding Correcting severe coagulation factor deficiencies before the procedure is indicated in hemophiliacs because they can experience intense local bleeding.

☑ CHECKPOINT 38-1

A 25-year-old male was recently diagnosed with acute lymphocytic leukemia (ALL) and received chemotherapy. It is 21 days after the chemotherapy. A bone marrow biopsy has been scheduled to determine whether residual leukemia is present. His platelet count is 10×10^9/L. Does this patient have a significant risk of bleeding? Is it necessary to cancel the procedure until the platelet count is $>50 \times 10^9$/L (50×10^3/mcL)?

BONE MARROW PROCESSING FOR EXAMINATION

A laboratory professional with experience in bone marrow preparation accompanies the physician, who performs the bone marrow aspirate and biopsy. The laboratory professional is responsible for assessing and processing the specimen so that it can be examined.

Bone Marrow Aspirate Smears, Particle Preparation, and Clot Sections

One of the laboratory professional's responsibilities is to determine the adequacy of samples. Approximately 1.0–1.5 mL of aspirate is needed to evaluate bone marrow morphology. Although each laboratory has its own preferences for processing the aspirate, a commonly used procedure is described here. Depending on the clinical setting, additional aspirate can be obtained for cytogenetics, flow cytometry, molecular studies, and cultures.

A small amount (about 0.5 mL) of the aspirate is placed on a glass slide and examined to determine whether bone marrow is present in the sample (sometimes it is blood rather than marrow). The presence of particles consisting of fat, granules, and/or small pieces of bone in the aspirate sample that are visible on the slide is evidence that the bone marrow cavity was entered and that marrow was effectively aspirated.[3] The laboratory professional immediately communicates the adequacy of the bone marrow aspirate to the physician. Bone marrow occasionally cannot be aspirated, resulting in a **dry tap**. This can be caused by inadequate technique or, more commonly, alterations in the bone marrow architecture such as extensive fibrosis (e.g., hairy cell leukemia, primary myelofibrosis) or very increased cellularity (e.g., leukemia). In these cases, bone marrow biopsy is especially important.

Direct smears of bone marrow aspirate are prepared at the bedside using a technique similar to that for preparing peripheral blood smears.[4] Organization of supplies and speed in making these smears is essential because the aspirate clots quickly. Bone marrow particle crush smears can be prepared by pouring a small amount of marrow aspirate onto a watch glass. Using a glass pipette, the marrow particles are transferred onto several glass slides. A second slide is placed gently on top of the slide with the drop containing the particles. The top slide is pulled apart to evenly distribute the marrow particles. Some laboratories mix a portion of the marrow aspirate with ethylenediaminetetraacetic acid (EDTA). This anticoagulated marrow is placed in a Wintrobe tube and centrifuged. The marrow is separated into four layers: fat and perivascular cells, plasma, buffy coat (containing myeloid cells), and red cells. The ratio between the fat/perivascular and the erythroid/myeloid layers reflects the overall marrow cellularity. Concentrate smears can be prepared from the buffy coat. The direct, concentrate, and particle crush smears are stained with Wright's stain. The clinical laboratory professional usually prepares several additional aspirate smears for special stains that can be necessary for classifying the acute leukemias and myelodysplastic syndromes and evaluating storage iron. The remaining bone marrow particles can be used for histologic evaluation.

For histologic evaluation, tissue particles admixed with blood can be left to clot and then fixed in 10% buffered formalin or B-5 (a fixative containing mercury) and processed for histologic

sectioning (clot sections). However, better results are obtained if the blood and particles are placed in an EDTA tube and mixed before clotting begins. The blood and particles are filtered through histowrap filter paper, and the concentrated particles on the paper are fixed in 10% buffered formalin. In the histology laboratory, the particles left on paper are collected and embedded in paraffin for further processing (particle preparation).[5] Sections are made of the paraffin-embedded clot section or particle preparation and stained with hematoxylin and eosin (H&E).

Touch Imprints and Core Biopsy

Immediately after obtaining the bone marrow core biopsy, touch imprints of the biopsy can be made by gently touching or rolling the tissue several times on 2–4 glass slides. Some cells from the core specimen stick to the slide. Some of these slides are stained with Wright's stain, and the rest can be used for special stains in case a good aspirate is not available. After making the touch preparations, the core biopsy specimen is immediately immersed in 10% buffered formalin fixative. The fixed trephine core biopsy undergoes histologic processing including decalcifying, dehydrating, embedding in paraffin blocks, sectioning of 2- to 3-mcM (μm) thickness, and histologic staining with H&E. Some laboratories prefer to fix bone marrow particles, clot, and core biopsy in B-5 first followed by 10% buffered formalin fixative. Although samples fixed in B-5 fixative have better morphologic sections than 10% formalin, B-5 fixative contains mercury and is environmentally unfriendly, and its disposal is difficult.

Trephine core biopsies are performed for primary diagnosis as well as for disease staging purposes. When performed for clinical staging of lymphoma, multiple myeloma, and carcinoma, bilateral biopsies are recommended.

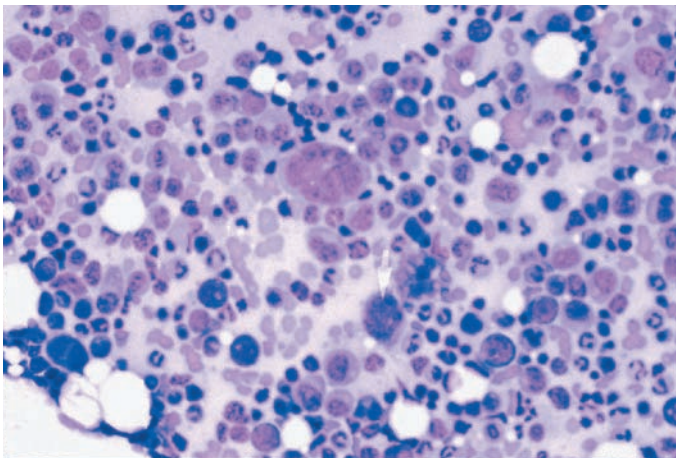
MORPHOLOGIC INTERPRETATION OF BONE MARROW

The morphologic review of bone marrow includes cytologic assessment of hematopoietic cells on the aspirate smear as well as histologic assessment of the clot, particle preparation, touch imprint, and core biopsy. Review of these preparations provides a wealth of information about hematopoiesis, including an estimation of cellularity, details of cell morphology, estimation of the quantity of iron and its distribution, and presence of abnormal cells such as tumor cells. It is good practice to review the complete blood count (CBC) data and the corresponding peripheral blood smear in conjunction with the bone marrow.[1] The number and appearance of the blood cells guide the morphologist to a differential diagnosis.

Bone Marrow Aspirate

Cellular morphology is best appreciated on well-stained aspirate smears. Thus, the aspirate smear is used to evaluate the number, morphology, and maturation process of erythroid and myeloid precursors and megakaryocytes (Figure 38-2 ■).

The bone marrow aspirate smears are scanned at 100× magnification to determine cellularity and to select a suitable area for examining and performing the differential count. Optimal areas are where the cells are spread out and intact. Areas where the marrow cells are destroyed due to squashing or stripping of their cytoplasm by fibrin threads should be avoided. These areas are characterized by the presence of bare nuclei.



■ **FIGURE 38-2** Bone marrow aspirate smear showing normal maturing trilineage hematopoiesis (bone marrow, Wright-Giemsa stain, 200× magnification).

Bone marrow cellularity is determined by estimating the percent of bone marrow space occupied by hematopoietic tissue. The reference range decreases with age; infants have the highest cellularity (90%) and older adults the lowest. While scanning for cellularity, the number and the distribution of the megakaryocytes are usually noted. Megakaryocytes are usually adjacent to a spicule, a fragment of spongy bone along which hematopoietic cells cluster and mature. An optimal aspirate has some spicules present, and most particles should contain at least one megakaryocyte. Direct smears should contain 5–10 megakaryocytes in the readable portion of the slide, and concentrate smears should contain numerous megakaryocytes.[3]

After the initial scan at 100× magnification, the details of nuclear and cytoplasmic maturation are evaluated at higher magnification (500× or 1000×). Evaluation of maturation is an important step when reviewing the marrow aspirate. Abnormal maturation can be seen in any of the three cell lineages. For example, abnormal maturation is a diagnostic criterion in the myelodysplastic syndromes (MDS). Abnormalities of the red cell precursors in MDS include multinucleation and nuclear/cytoplasmic asynchrony (nuclear maturation is delayed compared to cytoplasmic maturation). Myeloid precursors can show abnormal granule development, and megakaryocytes can reveal hypolobulation of the nucleus. Abnormalities in bone marrow that are characteristic of a specific pathology are discussed in Chapters 11–20 (anemias) and 23–28 (neoplastic disorders).

Bone Marrow Differential Count

A 500–1000 cell differential (a 500-cell differential can be performed on each of two slides) is usually performed by an experienced clinical laboratory professional or pathologist using 1000× magnification on the direct smear or the crush particle preparation (the morphology is better preserved on these preparations).[3] The concentrate and/or touch imprint is a useful source when the bone marrow is very hypocellular or an aspirate could not be obtained (dry tap); however,

the morphology is not as good as on direct smears and crush particle preparations. Overall marrow cellularity can be estimated on the particle preparation. The greatest mass of the adult marrow is composed of granulopoietic and erythropoietic precursors. For the purpose of the differential count, these hematopoietic cells are enumerated within different categories according to their stage of maturation.

Because the bone marrow composition changes with age, knowing the patient's age when evaluating bone marrow specimens is important. In infants during the first month after birth, dramatic alterations occur in the distribution of the different marrow components. Myeloid precursors are usually increased at birth followed (within a few weeks) by a predominance of lymphoid cells. These lymphoid cells are called **hematogones** and represent normal immature B cells[6] (Figure 38-3 ■). Differentiating hematogones from lymphoblasts is difficult, both morphologically and phenotypically. Hematogones show some immature features such as fine to smudged chromatin and a high nuclear-cytoplasmic ratio, but the morphology is variable, reflecting the hematogoines maturation to mature lymphocytes. In young children, lymphocytes can represent about one-third of marrow cellularity, which decreases gradually after puberty.

Bone marrow is not as uniformly homogeneous as blood. During the bone marrow procedure, the needle can be placed in an area that has clusters of erythroid or lymphoid cells. In adult bone marrow, lymphocytes are usually present as interstitial infiltrates and sometimes form lymphoid follicles, which are collections or aggregations of lymphocytes. These lymphocytes can be malignant or benign (see the section "Benign Lymphoid Aggregates versus Malignant Lymphoma"). Depending on the placement of the bone marrow needle, the cells in the follicle can be aspirated. This can give an estimate of markedly increased lymphocytes in the marrow and introduce significant variation in the differential count on different samples from the same patient.

Megakaryocytes are not included in the differential count but are evaluated by scanning the smear at 100×. Megakaryocytes are not evenly distributed in the marrow and on the aspirate smear are typically adjacent to a spicule.

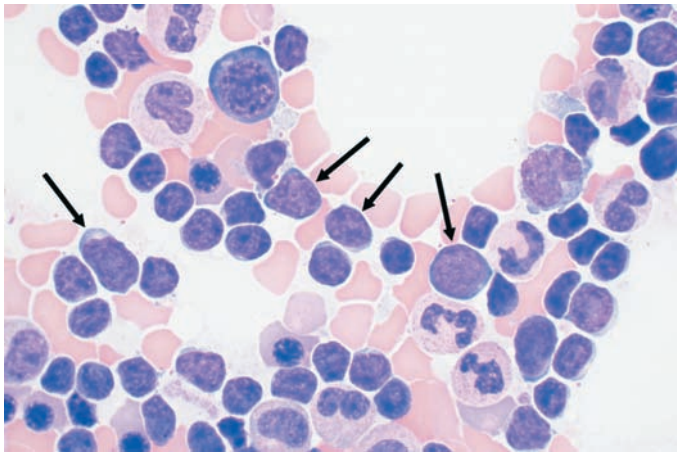
Cells not normally found in the peripheral blood, including macrophages, mast cells, osteoblasts, and osteoclasts, can be found in the bone marrow. However, these cells are not part of the bone marrow differential count. (These cells are described in Chapter 3.)

Myeloid to Erythroid Ratio

When adequate numbers of cells are counted (500–1000) and differentiated, the percentage of each category is calculated. The ratio between all granulocytes and their precursors and all nucleated red cell precursors represents the myeloid:erythroid ratio (M:E ratio). This parameter can be used to indicate significant changes in the cellularity of the myeloid and erythroid cells. The M:E ratio should always be interpreted in context with the overall cellularity. For instance, a low M:E ratio can indicate erythroid hyperplasia or myeloid hypoplasia. If it is known, however, that the bone marrow cellularity is increased, a low M:E ratio indicates erythroid hyperplasia or ineffective erythropoiesis. On the other hand, if the bone marrow cellularity is decreased, a low M:E ratio indicates myeloid hypoplasia.

The reference range for the M:E ratio differs, depending on whether the segmented neutrophils are included in the counts. If they are considered a part of the bone marrow storage pool but not included in the count, the M:E reference range is 1.5:1–3:1. If segmented neutrophils are included in the count, the M:E reference range is 2:1–4:1.

The granulocytic tissue occupies two to four times more marrow space than the erythrocytic precursors because of the shorter survival of the granulocytes in the circulation (i.e., neutrophils, 6–10 hours versus erythrocytes, 120 days). Changes in the survival time of granulocytes and erythrocytes are reflected in changes in the M:E ratio. The M:E ratio in hemolytic anemias can be decreased due to erythroid hyperplasia. Normal, increased, or decreased M:E ratios are associated with certain disease entities. For example, patients with chronic myelogenous leukemia usually have an increased ratio; in contrast, patients with MDS usually have a decreased ratio.



■ **FIGURE 38-3** A bone marrow aspirate smear from a 21-year-old woman following chemotherapy for acute lymphoblastic leukemia (ALL). Hematogones typically display homogenous chromatin with a smudged appearance, indistinct nucleoli, and scant cytoplasm (arrows). Many hematogones are indistinguishable from ALL blasts, but the morphology of hematogones is variable, reflecting the maturation of the hematogones toward mature lymphocytes (Wright stain, 1000× magnification).

☑ CHECKPOINT 38-2

A 60-year-old male complained to his local physician of abdominal fullness. The CBC showed an elevated leukocyte count with absolute neutrophilia and basophilia. The peripheral blood smear revealed numerous myelocytes. A bone marrow biopsy was performed. The bone marrow differential was reported as follows: blasts 1%, myelocytes 35%, metamyelocytes 20%, bands 10%, segmented neutrophils 20%, eosinophils 1%, basophils 3%, pronormoblasts 1%, basophilic normoblasts 3%, polychromatophilic normoblasts 2%, orthochromatic normoblasts 4%, lymphocytes 0%, and monocytes 0%. What is the M:E ratio?

Touch Imprints

When the bone marrow is a dry tap secondary to underlying fibrosis (e.g., myelofibrosis, hairy cell leukemia) or when the marrow aspirate lacks spicules and is hemodiluted, the touch imprints of the bone marrow biopsy can be the only source for studying the sample's cellular detail and maturation sequence. Sometimes the touch preparations contain enough cells for performing a differential count and cytochemical stains (Figure 38-4 ■).

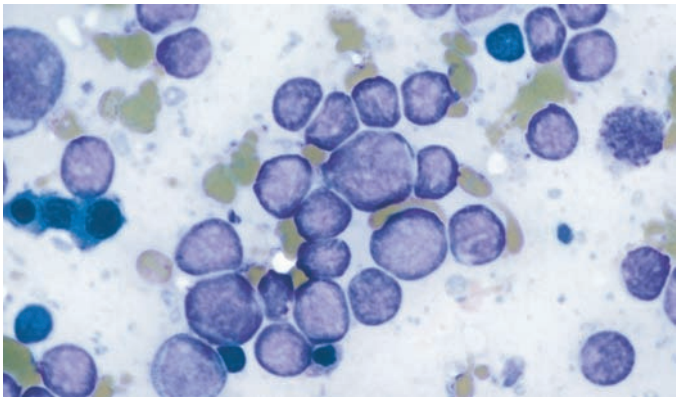
Bone Marrow Particle Preparation and Clot and Core Biopsy

The advantage of the bone marrow biopsy is that it represents a large sample of marrow and bone structures in their natural relationship. Some marrow particles obtained during the aspiration procedure and the biopsy specimen can be processed for histologic examination. An adequate bone marrow core biopsy should be >1 cm in adults. Clot and particle preparation sections should contain fragments of recognizable bone marrow tissue.

In addition to routine hematoxylin and eosin stains, various other stains also can be used. An iron stain can be performed to evaluate storage iron; however, because of decalcification, the core biopsy might not be good for studying iron in marrow. Aspirate smears, particle preparations, or clot sections should be used to evaluate iron stores. Reticulin and trichrome stains are used to evaluate the presence of fibrosis. Identification of a granuloma (a chronic inflammatory reaction in which the predominant cell type is an activated macrophage with an epithelial-like appearance) can assist in the diagnosis of granulomatous diseases including infectious diseases. Other conditions associated with granulomas include lymphoma and malignancies. Many drugs have been implicated in the genesis of granulomas.[7] Acid-fast organisms and fungi in **granulomatous** diseases can be detected quickly with specific stains offering great advantages in diagnosing these infections. When metastatic tumors and lymphomas are found in the bone marrow, **immunohistochemical stains** can be used on histologic sections to demonstrate specific tumor markers. Immunohistochemical stains use labeled antibodies to identify specific markers (antigens) on cells. Thus, a precise diagnosis of the origin of a tumor can be made without elaborate, expensive, and more invasive techniques. Immunophenotyping by flow cytometry can be performed only on fresh unfixed bone marrow samples (aspirate or core biopsy); however, immunophenotyping by immunohistochemistry is performed on fixed, paraffin-embedded samples.

Molecular studies can be done on marrow specimens to aid in diagnosing and managing some diseases. Polymerase chain reaction (PCR) can be performed on fresh samples and paraffin-embedded formalin fixed marrow biopsies. PCR technology can be used to detect B-cell or T-cell gene rearrangements, various leukemias, and minimal residual disease and to diagnose infectious diseases (Chapter 42).

A disadvantage of the bone marrow biopsy is that it loses fine cellular details in processing; therefore, it is of little value in diagnosing leukemias and some refractory anemias. In these situations, the touch preparation from the biopsy could supply the missing morphologic details.



■ **FIGURE 38-4** Touch imprint from a case with ALL showing many lymphoblasts. Marrow was inaspirable and therefore was not available for the morphologic review. Cytochemical stains were performed on these imprints, and blasts were negative for all stains. (BM touch imprints, Wright-Giemsa stain, 500× magnification).

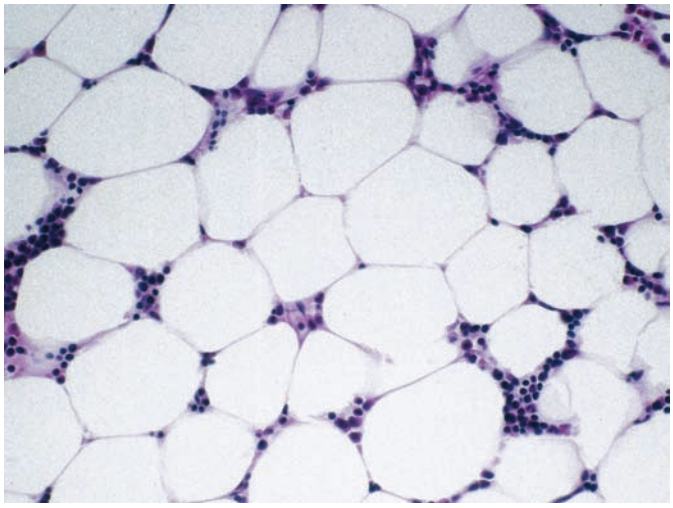
Bone Marrow Cellularity

The overall bone marrow cellularity is estimated by comparing the amount of hematopoietic tissue with the amount of adipose (fat) tissue. Fat appears as a clear space in the marrow sample. An easy way to determine the normal expected cellularity range in an adult is to subtract the patient's age from 100% ± 10%. For example, a normal 70-year-old adult has an overall cellularity between 20% and 40%. Variation in cellularity within the bone marrow can occur. The subcortical and paratrabecular areas are more hypocellular than the deeper medullary area, and variability occurs in cellularity within the sample itself. For these reasons, estimated cellularity is based on an average percentage. If both hypercellular and hypocellular areas are present within the medullary area it should be described in the report because the average cellularity can be difficult to estimate.

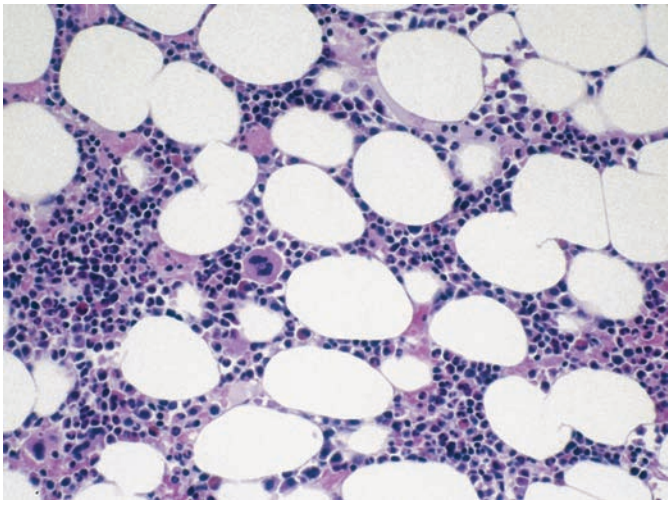
Although cellularity can be estimated on aspirate smears, clot sections, and particle preparations, the bone marrow biopsy is the optimal sample for this purpose and is typically evaluated at 10× magnification. After considering the patient's age, bone marrow cellularity is reported as decreased (less than the expected number of hematopoietic cells), normal, or increased (more than the expected number of cells). With experience, reproducible and reliable results can be achieved when evaluating marrow cellularity (Figure 38-5 ■).

Benign Lymphoid Aggregates versus Malignant Lymphoma

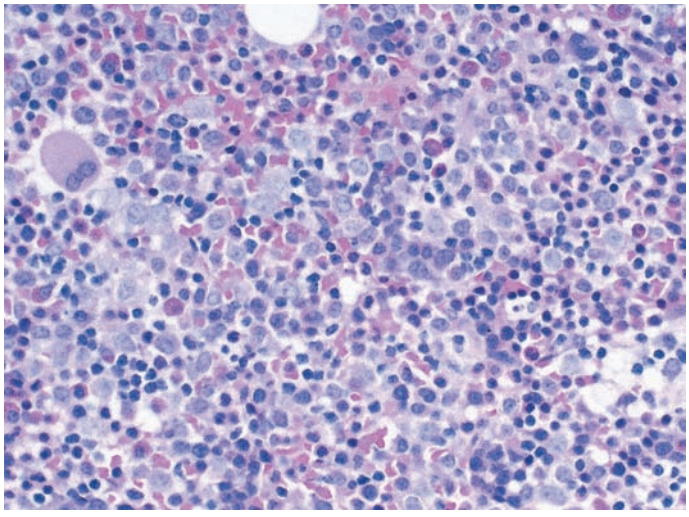
Reactive lymphoid aggregates can be seen in the bone marrow biopsy of elderly individuals. When one sees these aggregates, distinguishing between reactive (benign) versus lymphoma (malignancy) becomes important. Features suggestive of benign aggregates are nonparatrabecular, single, small, well-defined, polymorphic populations of lymphocytes (small and large lymphocytes) with plasma cells at the periphery and presence of blood vessels within the aggregate (Figure 38-6 ■). In contrast, lymphoid aggregates in lymphoma are typically ill defined, small to large and composed of monomorphic cells. The malignant aggregates can be paratrabecular or interstial, and can be diffuse or patchy (Figure 38-7 ■). If in doubt,



a



b



c

FIGURE 38-5 These bone marrow biopsies are from 40-year-old patients. Shown are **(a)** hypocellular marrow, **(b)** normocellular marrow, and **(c)** hypercellular marrow (bone marrow; paraffin section, hematoxylin-eosin stain; 100× magnification).

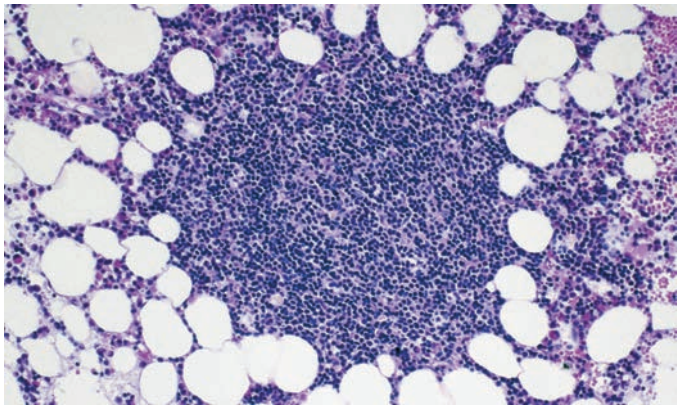


FIGURE 38-6 Bone marrow biopsy showing a well-circumscribed lymphoid aggregate indicating a benign lymphoid aggregate (bone marrow; paraffin section, hematoxylin-eosin stain; 100× magnification).

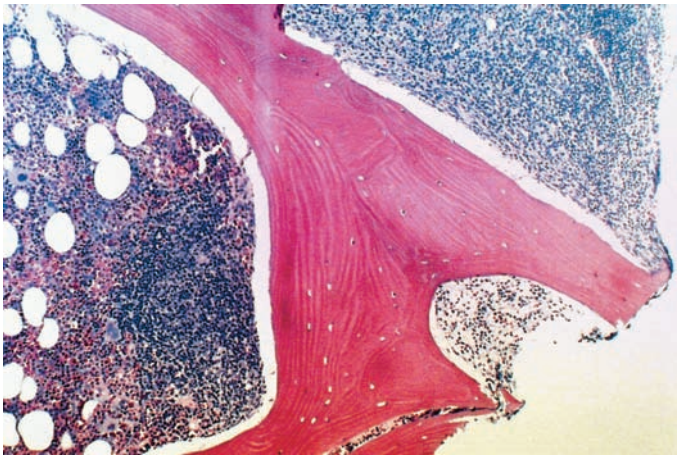


FIGURE 38-7 Bone marrow biopsy showing two paratrabecular lymphoid infiltrates suggesting involvement of marrow by a follicle center cell lymphoma (Bone marrow; paraffin section, hematoxylin-eosin stain; 100× magnification).

immunohistochemical stains and/or PCR can be performed on the marrow biopsy specimen to differentiate between benign lymphoid aggregates and lymphoma. The presence of an aberrant phenotype or clonal B or T cells would favor the diagnosis of lymphoma involving the bone marrow.

Bone Marrow Iron Stores

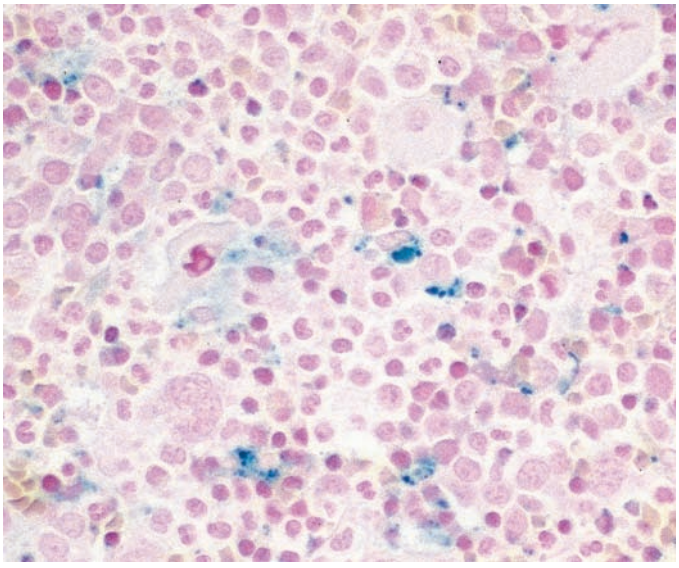
Iron is stored as ferritin (iron complexed with apoferritin protein) and hemosiderin in histiocytes (marrow macrophages) and erythroblasts. The storage iron in the bone marrow that can be visualized without specific iron stains is in the form of hemosiderin. On Wright-Giemsa-stained smears, hemosiderin appears as brownish blue granules, whereas it is golden yellow on unstained smears. To precisely evaluate intracellular storage iron, slides are usually stained with a Prussian blue stain.

Prussian Blue Stain for Iron

The Prussian blue stain provides the most direct means for assessing body iron stores. It is designed to demonstrate the presence of hemosiderin in histiocytes, erythroblasts (sideroblasts), and/or erythrocytes (siderocytes). EDTA chelation should be used to decalcify the bone marrow biopsies if iron studies are being performed. Rapid acid decalcifying solutions extract iron; therefore, these specimens must not be used.

In the Prussian blue staining procedure, a weak acid solution frees the iron from the loose protein bonds present in the hemosiderin molecule. This free iron combines with potassium ferrocyanide to produce ferric ferrocyanide. Free iron appears greenish-blue.

Histiocytes contain marrow iron that is usually seen as fine cytoplasmic granules. Sideroblasts are marrow erythroid precursors that contain iron specks (Figure 38-8 ■). Storage iron can be reported as absent, decreased, adequate, or increased. Some institutions give a numerical value of 0–4 (2 representing adequate iron stores in an adult).



■ **FIGURE 38-8** Bone marrow clot section stained with Prussian blue. The bluish granules indicate the presence of iron (bone marrow; clot section, Prussian blue stain; 400× magnification).

Evaluating and grading iron stores is subjective; thus, for consistency and precision, some guidelines should be followed such as increased storage when clumps of iron are easily seen at 100× magnification; a few specks of iron found after looking at several microscopic fields at 500× or 1000× magnification indicate decreased iron storage. When no stainable iron is detected in the bone marrow smear or tissue sections, iron is depleted or absent. Iron is also stored as intracellular ferritin. All developing normoblasts and reticulocytes contain some dispersed ferritin but it may not stain with Prussian blue. Small amounts of ferritin enter the blood and can be measured as serum ferritin (Chapter 12). The amount of circulating ferritin usually parallels the concentration of storage iron in the body. Nonspecific increases are associated with chronic infection and inflammation, liver disease, and malignancy. Thus, serum ferritin is not as accurate an indicator of iron sufficiency as hemosiderin in the presence of these conditions.

Use of Bone Marrow Iron Evaluation

Evaluating marrow iron stores is essential in diagnosing certain anemias, especially in refractory and dyserythropoietic anemias. When the morphologic characteristics of iron particles in the histiocytes and red cell precursors are an important diagnostic consideration (i.e., the presence of ringed sideroblasts), an iron stain can be performed on a particle smear. If the overall distribution of the amount of iron is of clinical importance (iron deficiency anemia, anemia of chronic diseases), histologic sections of bone marrow particles and marrow clotted particles are stained for iron.

The determination of bone marrow storage iron is useful in classifying anemias associated with defective hemoglobin synthesis. Storage iron is markedly decreased or absent in iron-deficiency anemia (Chapter 12). When storage iron is present in the bone marrow, anemia cannot be a result of iron deficiency unless the patient has been transfused with red cells or treated with iron supplements. Iron stores are normal or increased in anemia of chronic disease and thalassemias. Iron stores are also increased in sideroblastic anemia. The presence of **ring sideroblasts** is used in making the diagnosis of sideroblastic anemia and myelodysplastic syndromes. Ring sideroblasts are defined as erythrocyte precursors that contain at least five iron-containing particles, encircling one-third or more of the nucleus. This iron is located within the mitochondria that surround the nucleus. Ring sideroblasts can also be seen in myeloproliferative disorders, megaloblastic anemia, alcoholism, postchemotherapy, and some toxic exposures.

☑ CHECKPOINT 38-3

A pathologist is evaluating a bone marrow biopsy from a patient suspected of having a myelodysplastic syndrome. Evaluation of the peripheral blood reveals both hypochromic and normochromic red cells. The bone marrow aspirate shows dysplastic (abnormal cell development) changes in the red cell precursors. The pathologist ordered an iron stain on the particle crush preparation. Why is the iron stain useful in this case?

SPECIAL STUDIES ON BONE MARROW

In most cases, a thorough examination of a well-stained bone marrow aspirate smear and core biopsy provides enough information to diagnose hematopoietic disorders. However, several ancillary studies can provide additional information that help support or confirm the diagnosis. These studies can also provide prognostic information and, in some cases, can predict response to therapy. The most commonly used ancillary studies are flow cytometry, conventional cytogenetics, fluorescent in situ hybridization (FISH), molecular genetic studies, and cytochemical stains (Chapters 37, 40–42). Triaging the bone marrow samples for these tests depends on what clinical answer is being considered and thus can vary case by case. A guideline in Table 38-2 ★ can be modified in any lab, depending on the need and/or protocols.

Flow Cytometry

Flow cytometry allows the simultaneous analysis of multiple characteristics of cells in suspension as they pass through a laser light beam (Chapter 40). The size and internal complexity of the cells can be estimated, and with the use of fluorochrome-labeled antibodies, the presence of specific cell markers can be determined. Flow cytometry has a wide range of uses in evaluating hematologic disorders and is commonly used to establish the presence of clonality, diagnose and classify malignant lymphomas and leukemias, and detect minimal residual disease after chemotherapy. The immunophenotyping of peripheral blood, bone marrow, lymph node (and other) tissues, and body fluids is performed only on fresh samples (Table 38-3 ★) (Chapter 40).

Cytogenetics

Chromosome analysis is frequently used in the study of hematopoietic disorders because certain malignancies have characteristic chromosomal alterations (Chapter 41). In addition, the presence of some specific chromosomal abnormalities has prognostic implications. Chromosome analysis is routinely used in the primary diagnosis of acute leukemias, malignant lymphomas, MDS, and myeloproliferative neoplasms (MPNs). Because lymphoma cells are more difficult to grow, adequate clinical history needs to be provided when the sample is sent to the cytogenetics laboratory to allow the cytogenetic technologist to make procedural adjustments. Analysis is performed on fresh samples (Table 38-3). More recent techniques such as FISH have allowed specific cytogenetic questions to be answered more quickly.

Molecular Genetics

Although molecular genetic techniques were initially used in research laboratories, they are now performed in clinical laboratories. For hematolymphoid disorders, PCR has emerged as the leading adjunctive technology. The PCR method is used to demonstrate cell clonality and lineage and to identify specific genetic rearrangements. Additionally, PCR can be used to detect minimal residual disease (Chapter 42). Fresh and frozen samples are preferable for PCR; however, samples fixed in formalin or embedded in paraffin are acceptable (Table 38-3).

★ **TABLE 38-2** Triaging Bone Marrow Samples for Ancillary Studies

Diagnosis/History	Flow Cytometry	Cytogenetics/FISH	Molecular Studies
Acute leukemia (new, follow-up or after stem cell transplant)	Yes	Yes	Maybe[a] After stem cell transplant—chimerism studies can be performed by PCR
CLL	Yes	Yes	No
Chronic myeloproliferative neoplasms	No	Yes	Yes (*JAK-2* mutation)
Polycythemia vera			
Essential thrombocythemia			
Chronic idiopathic myelofibrosis			
CML	No[b]	Yes	Yes (*BCR/ABL1* mutation)
CMML	Yes	Yes	No[c]
Myelodysplasia	No[b]	Yes	No
Multiple myeloma	No	Yes	No
Hodgkin's lymphoma	No	No	No
Non-Hodgkin's lymphoma	Yes	Maybe[d]	Maybe[d]

[a]Molecular studies are usually not needed (depends upon the subtype of acute leukemia) because most genetic abnormalities seen in acute leukemias can be detected by cytogenetics/FISH. However, the distinction between p210 and p190 chimeric protein in ALL with t(9;22) is made by molecular analysis. Storing cell pellet with the possibility of doing the assay if needed is a good idea if there is uncertainty about submitting for molecular studies.
[b]If blasts are increased or CML in blast crisis is suspected, send for flow cytometry.
[c]Some cases of CMML could need molecular or FISH studies (for example, if there is suspicion of t(5;12) and the cytogenetics are normal).
[d]When suspecting certain lymphomas (for example, Mantle cell, Burkitt/Burkitt-like versus large cell lymphomas or anaplastic large cell lymphoma), send the sample for cytogenetics and/or FISH. When suspecting follicle center cell lymphoma, send it to molecular lab for PCR or FISH for Bcl-2 rearrangement.
This table can be modified in any lab, depending on the need and/or protocols.

PCR = polymerase chain reaction; CLL = chronic lymphocytic leukemia; CML = chronic myelogenous leukemia; *BCR/ABL1* = fusion gene located in the Philadelphia (Ph) chromosome (#22); *JAK-2* = Janus kinase 2; CMML = chronic myelomonocytic leukemia; FISH = fluorescence in situ hybridization; ALL = acute lymphocytic leukemia.

★ **TABLE 38-3** Specimen Requirements for Special Studies on Bone Marrow

Specimen	Flow Cytometry	Cytogenetics	Molecular Studies
Blood	5 mL in EDTA, heparin sodium, or ACD	5 mL preferably in heparin sodium	1–3 mL in EDTA
Bone marrow	1 mL in EDTA, heparin sodium, or ACD	1–3 mL preferably in heparin sodium; bone marrow preferable over blood	1–3 mL in EDTA (preferable over heparin)
Fluids	As much as possible, anticoagulated if contaminated with blood	As much as possible, anticoagulated if contaminated with blood	5–10 mL
Tissue	0.5 cm^2 fresh in transport media or saline	1 cm^2 fresh in transport media	3–5 mm^2 fresh or frozen; fixed specimens good for PCR analysis
All specimens: transport and storage	Should be kept at room temperature and analyzed within 24 hours; up to 72 hours is acceptable in some cases; should be refrigerated if analysis will not occur within 24 hours; frozen or fixed specimens unacceptable	Should be kept at RT and transported to the laboratory within 24 hours; frozen or fixed samples unacceptable	If not analyzed within 24 hours, should be refrigerated (2–8°C) for up to 1 week; samples frozen at −20°C or −70°C should be stored for 2 weeks or 2 years, respectively

ACD = acid citrate dextrose; EDTA = ethylenediaminetetraacetic acid; RT = room temperature

CASE STUDY *(continued from page 817)*

A bone marrow procedure was performed. The marrow was difficult to aspirate, but the physician was able to obtain some hemodiluted marrow. Bilateral marrow biopsy was also performed. Review of the aspirate smears showed a very hemodiluted sample, but the pathologist was able to see an increased number of blasts. There was not enough aspirate sample to do flow cytometry or cytogenetic testing and cytochemical stains.

2. How should the marrow evaluation proceed?

Cytochemical Stains

In hematology, *cytochemistry* refers to staining cells in vitro to allow microscopic examination of the cells' chemical composition. Cells of different lineages have different cytochemical compositions. The staining process does not significantly alter cell morphology. Cytochemical stains are usually performed on specimens from patients who have a neoplasm of hematopoietic cells. These stains help differentiate the lineage of immature cells so that an accurate diagnosis of the neoplasm type can be made. (Staining procedures and results for cell lineages are described in Chapter 37; see Table 37-12.)

Smears are usually made from peripheral blood and bone marrow. Although the bone marrow aspirate is usually used to make smears for cytochemical stains, the touch imprints from the biopsy can be used if the aspirate is insufficient. The smears are incubated with substrates that react with specific cellular constituents (organelle-associated enzymes, carbohydrates, and proteins). If the specific constituent is present in the cell, its reaction with the substrate is confirmed by the formation of a colored product within the cell. The stained slides are evaluated with an ordinary light microscope. Most stains can be performed on air-dried smears. All cytochemical stains should be performed on recently prepared slides. Some cellular constituents are sensitive to heat, light, storage, and processing technique. If stains cannot be performed in a timely fashion, the unstained smears can be protected from light and stored in a refrigerator. However, a fresh smear is preferred for the myeloperoxidase (MPO) stain (Chapter 37). (Many laboratories no longer do cytochemical stains but identify cells using flow cytometry.)

BONE MARROW REPORT

The bone marrow report must contain all relevant information and optimally is composed of two components: clinical information and morphologic interpretation. The clinician should provide the patient's biographic data, clinical differential diagnosis, and relevant therapeutic information. The morphology and interpretation by the pathologist and laboratory professional must include site of sampling (e.g., sternum), types of sample obtained, differential counts from both peripheral blood and bone marrow, and morphologic abnormalities in any cell lineages in the patient's peripheral blood or bone marrow. The results must be interpreted in conjunction with any additional studies (special stains, flow cytometry, cytogenetics, molecular studies). If the additional studies are significant in establishing the diagnosis but are unavailable when the bone marrow report is written, an addendum report should mention the significance of these studies. The comparison of the current marrow specimen to the previous tissue samples (marrow or other nonmarrow biopsies) is essential in some situations.

Finally, the pathologist should render a diagnostic interpretation within a reasonable period of time. For example, if 50% blasts were present in the blood or the bone marrow, it is reasonable to call the clinician about the preliminary diagnosis of acute leukemia. However, the final diagnosis can be made only after the special stains and flow cytometry studies have been completed. Comments, if needed, should be concise and relevant to the case and can include a recommendation for additional tests and a possible differential diagnosis.

CASE STUDY

Processing bone marrow biopsy usually is done overnight.

3. Can a diagnosis be made from tests on the aspirate rather than waiting for the biopsy slides?

Summary

The bone marrow is the primary site of blood cell production after birth. Evaluation of bone marrow is indicated in the diagnosis, management, and follow-up of a variety of hematopoietic disorders including some anemias and malignancies. Bone marrow specimens include aspirates and biopsy. The marrow is processed and evaluated in the laboratory.

Differentiation and classification of hematologic neoplasms depend on accurate identification of the malignant cells. Because the lineage of the cells is difficult to differentiate using only morphologic characteristics, immunophenotyping by flow cytometry and/or immunohistochemistry (and cytochemical stains) are used to help identify the lineage. Cytogenetic analysis is commonly used in diagnosis because certain malignancies have characteristic chromosomal abnormalities. Molecular analysis can be used to demonstrate clonality, cell lineage, and genetic abnormalities. It also is useful in diagnosing and evaluating minimal residual disease.

Review Questions

Level I

1. What is the site for bone marrow collection in children under 2 years of age? (Objective 1)
   A. spleen
   B. sternum
   C. tibia
   D. lumbar vertebral bodies
2. Which of the following indicates that a bone marrow biopsy should be performed? (Objective 2)
   A. 20-year-old male with suspected leukemia
   B. 60-year-old female diagnosed with megaloblastic anemia
   C. 30-year-old male diagnosed with HIV
   D. 20-year-old pregnant woman diagnosed with iron-deficiency anemia
3. What site is most commonly used in adults for bone marrow biopsies? (Objective 1)
   A. sternum
   B. posterior iliac crest
   C. anterior iliac crest
   D. tibia
4. The marrow specimen that is best for preserving the architecture of the bone marrow is: (Objective 3)
   A. aspirate
   B. clot section
   C. particle smear
   D. biopsy
5. Which of the following is the best specimen for evaluating the morphology of the hematopoietic precursors? (Objectives 3, 4)
   A. bone marrow aspirate
   B. clot preparation
   C. iliac crest biopsy
   D. touch imprint
6. Which of the following diseases is more likely to have a decreased M:E ratio? (Objective 5)
   A. chronic blood loss
   B. granulocytic leukemia
   C. lymphocytic leukemia
   D. chronic infection
7. What is the expected overall cellularity in a bone marrow biopsy of a normal 50-year-old male? (Objective 6)
   A. 80% ± 10%
   B. 100% ± 10%
   C. 50% ± 10%
   D. 30% ± 10%
8. Which of the following stains is used to evaluate the presence of storage iron? (Objective 7)
   A. Wright-Giemsa
   B. Prussian blue
   C. PAS
   D. reticulin

9. Why would special stains be performed on a bone marrow specimen? (Objective 9)
   A. to differentiate leukemias
   B. to determine the M:E ratio
   C. to estimate bone marrow cellularity
   D. to identify hematogones
10. The clinical laboratory professional's role in bone marrow evaluation is to: (Objective 8)
   A. make direct bone marrow smears
   B. perform the bone marrow aspirate procedure
   C. administer the anesthetic before the procedure
   D. determine the appropriate therapy

Level II

1. Which of the following is characteristic of a benign lymphoid aggregate? (Objective 1)
   A. The patient has a history of lymphoma.
   B. It is composed predominantly of polymorphic lymphocytes.
   C. It is large with ill-defined borders.
   D. It is paratrabecular and lacks plasma cells at the periphery.
2. A hypercellular bone marrow M:E ratio is 10:1. This indicates: (Objective 3)
   A. decreased erythropoiesis
   B. increased erythropoiesis
   C. increased leukopoiesis
   D. decreased leukopoiesis
3. The following is the differential obtained from a patient with a diagnosis of AML: blasts 65%, myelocytes 4%, metamyelocytes 4%, bands 1%, segmented neutrophils 8%, pronormoblasts 2%, basophilic normoblasts 3%, polychromatic normoblasts 3%, orthochromatic normoblasts 3%, lymphocytes 4%, and monocytes 3%. What is the M:E ratio? (Objective 3)
   A. 10:1
   B. 5.4:1
   C. 7.4:1
   D. 1:7.4
4. When evaluating bone marrow biopsy for estimating the marrow cellularity, which ratio is being estimated? (Objective 4)
   A. hematopoietic cells to fat
   B. hematopoietic cells to bony trabeculae
   C. hematopoietic cells to vessels
   D. granulocytes to fat
5. A pathologist is evaluating a bone marrow biopsy from a patient recently diagnosed with lymphoma. The bone marrow evaluation should include investigation for which of the following? (Objective 1)
   A. malignant lymphoid aggregate
   B. granulomatous lesions
   C. iron stores
   D. presence of parasites
6. A pathologist is evaluating a bone marrow biopsy in a patient recently diagnosed with Hodgkin lymphoma. Inadvertently, the lab performed an iron stain on a particle crush preparation. The pathologist saw a few ring sideroblasts but did not see any dysplastic changes suggesting myelodysplastic syndrome. In which of the following conditions might ring sideroblasts appear? (Objective 5)
   A. iron-deficiency anemia
   B. thalassemia
   C. alcoholism
   D. Hodgkin's disease
7. A clinical laboratory professional evaluating the bone marrow particle preparation of a patient suspected of having acute leukemia identifies a large number of undifferentiated blasts. What ancillary test should be done? (Objective 2)
   A. molecular diagnostics
   B. cytochemical stains
   C. fluorescent in situ hybridization
   D. HIV testing
8. A physician performs a bone marrow biopsy and aspirate on a 65-year-old male. The cellularity is estimated at 80%, and the M:E ratio is 0.5:1. The correct description of this marrow is: (Objectives 3, 4)
   A. hypercellular with a decreased M:E ratio
   B. hypercellular with an increased M:E ratio
   C. hypocellular with a decreased M:E ratio
   D. hypocellular with an increased M:E ratio

9. A physician performing a bone marrow biopsy on a patient with a possible diagnosis of acute leukemia is unable to obtain any aspirate but did obtain two core biopsies. What is the best way for the clinical laboratory professional to use the material obtained? (Objectives 2, 6)

   A. Place both core biopsies in formalin and process one immediately; save the other for ancillary tests.

   B. Place one core biopsy in formalin and freeze the second one at −70°C.

   C. Place one core biopsy in formalin after making touch imprints; put the second in saline.

   D. Place both core biopsies in saline; freeze one at 0°C and process the other immediately.

10. A 20-year-old patient presented with severe headache. A lumbar puncture performed showed a marked increase in atypical mononuclear cells. The differential diagnosis is acute leukemia or malignant lymphoma. Which of the following would be most helpful in distinguishing the two diagnoses? (Objective 2)

    A. molecular diagnostic tests

    B. flow cytometry

    C. cytogenetics tests

    D. cytochemical stains

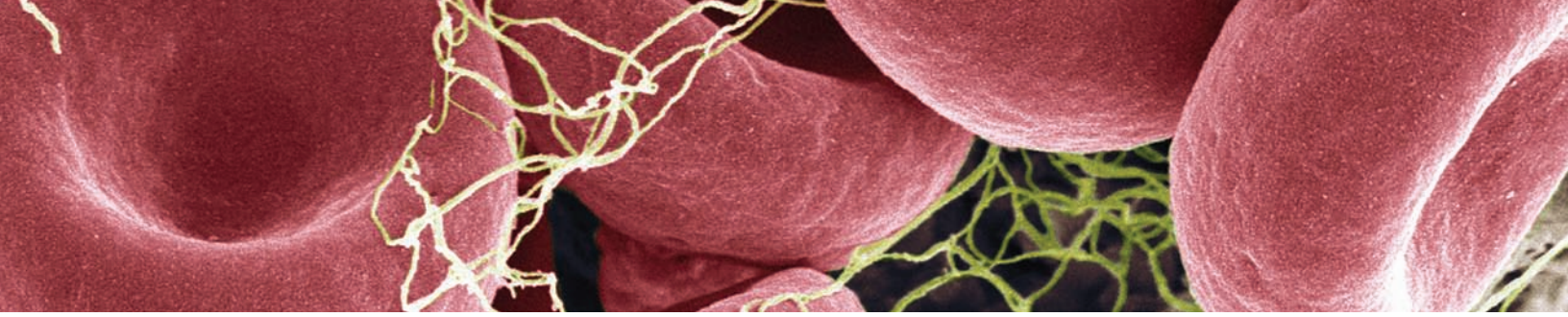
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Hutchinson RE, McPherson RA, Schexneider KI. Basic examination of blood and bone marrow. In: McPherson RA, Pincus MR, eds. *Henry's Clinical Diagnosis and Management by Laboratory Methods*, 22nd ed. Philadelphia: Elsevier; 2011.
2. Jamshidi K, Swaim WR. Bone marrow biopsy with unaltered architecture: a new biopsy device. *J Lab Clin Med*. 1971;77:335–42.
3. Ryan DH, Cohen HJ. Bone marrow examination. In: Hoffman R, Benz EJ Jr, Shattil SJ et al., eds. *Hematology Basic Principles and Practice*, 4th ed. Philadelphia: Elsevier Churchill Livingstone; 2005.
4. Rywlin AM, Marvan P, Robinson MJ. A simple technique for the preparation of bone marrow smears and sections. *Am J Clin Pathol*. 1970;53:389–93.
5. Peterson LC, Brunning RD. Bone marrow specimen processing. In: Knowles D, ed. *Neoplastic Hematopathology*, 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 2001.
6. Longacre TA, Foucar K, Crago S et al. Hematogones: a multiparameter analysis of bone marrow precursor cells. *Blood*. 1989;73:543–52.
7. Eid A, Carion W, Nystrom JS. Differential diagnosis of bone marrow granuloma [review]. *West J Med*. 1996;164(6):510–15.



39 Automation in Hematology

CHERYL BURNS, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Cite the electrical impedance principle of cell counting, and identify the instruments that use this technology.
2. Describe the use of radio frequency in cell counting, and identify the instruments that use this technology.
3. State the principles of light scatter used in cell counting, and identify the instruments that use this technology.
4. List the reported parameters for each blood cell–counting instrument.
5. Categorize cell parameters as directly measured and derived from a histogram, scattergram or cytogram, or calculation.
6. Describe the principle of reticulocyte count enumeration by automated blood cell–counting instruments.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Compare and contrast the methods of analysis for the described histograms, scatterplots, scattergrams, and cytograms, and interpret the results.
2. Describe and interpret the automated reticulocyte parameters.

Key Terms

Aperture
Cellular hemoglobin concentration mean (CHCM)
Cluster analysis
Coincidence
Continuous flow analysis
Contour gating
Hemoglobin distribution width (HDW)
Histogram
Hydrodynamic focusing
Isovolumetric sphering
Mean platelet volume (MPV)
Reagent blank
Reticulated platelet
Scatterplot
Threshold limit
Viscosity

Chapter Outline

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before beginning this unit of study:

Level I

- State the principles of cell enumeration by hemacytometer. (Chapter 37)
- State the principle of the cyanmethemoglobin method for hemoglobin determination. (Chapter 37)
- Calculate the erythrocyte indices. (Chapters 10, 37)
- Describe the peripheral blood smear examination process and correlate peripheral blood smear findings with complete blood counts (CBCs). (Chapters 10, 37)
- Describe and state the principle of the manual reticulocyte count procedure. (Chapter 37)

Level II

- Summarize the diagnostic use of the reticulocyte count in evaluating hematologic disorders and monitoring various therapies such as iron replacement and bone marrow transplant. (Chapters 11, 12, 29)

OVERVIEW

Automation is firmly established within the hematology laboratory. This chapter reviews the fundamental principles of the hematology instruments and describes examples of instrumentation that are currently used. The basic principles of operation are discussed, and samples of normal test results are displayed for each instrument.

INTRODUCTION

The evolution of instrumentation in hematology began in the mid-1950s. Until that time, laboratory professionals performed manual hemacytometer blood cell counts, spun hematocrits, spectrophotometrically determined hemoglobins, and microscopic blood smear evaluations. With the advent of the first automated blood cell–counting instrument, manual hemacytometer blood cell counts for erythrocyte and leukocyte enumeration were replaced. In general, automated blood cell–counting instruments provide data with increased reliability, precision, and accuracy.

AUTOMATED BLOOD CELL–COUNTING INSTRUMENTS

With the many advances in hematology instrumentation, automation currently encompasses the primary testing in the hematology laboratory. Automated instruments can perform a complete blood count (CBC), including a five-part leukocyte differential and absolute reticulocyte count as well as certain body fluid cell counts (e.g., cerebrospinal fluid white blood cell [WBC] count). A number of principles for cell counting and differential analysis have been used in the past. The two major principles of blood cell counting currently used by the hematology instruments are impedance and optical light scattering.

The impedance principle of blood cell counting is based on the increased resistance that occurs when a blood cell with poor conductivity passes through an electrical field. The number of pulses indicates the blood cell count and the amplitude (i.e., height) of each pulse is proportional to the volume of the cell.[1] Examples of instruments using this principle are offered by Beckman Coulter, Inc., Sysmex Corporation, and Abbott Diagnostics.

The optical light-scattering principle of blood cell counting is based on light-scattering measurements obtained as a single blood cell passes through a beam of light (optical or laser). Blood cells create forward scatter and side scatter that photodetectors detect. The degree of forward scatter is a measurement of cell size, and the degree of side scatter is a measurement of cell complexity or granularity.[2] The Siemens Healthcare instruments use this principle for all blood cell counts, and newer models manufactured by Sysmex Corporation and Abbott Diagnostics use this principle for leukocyte enumeration and differentials.

The automated reticulocyte count has essentially replaced the manual reticulocyte count for evaluating the bone marrow's erythropoietic activity. Among the hematology instruments, the method of reticulocyte enumeration varies and is discussed for each instrument. These automated methods determine additional reticulocyte parameters including reticulocyte hemoglobin concentration (CHr™ or Ret He), reticulocyte mean cell volume (MCVr), and immature reticulocyte fraction (IRF). The reticulocyte hemoglobin concentration is determined by directly measuring the reticulocyte's hemoglobin by light-scattering characteristics. The reticulocyte mean cell volume is derived from the reticulocyte cytogram and reflects the average size of the reticulocyte population. The immature reticulocyte fraction, or high-intensity ratio, reflects reticulocytes with an increased amount of ribonucleic acid (RNA) or early immature reticulocytes. The presence of increased numbers of immature reticulocytes can be an indicator of an erythropoietic response. The reticulocyte hemoglobin concentration is an assessment of iron incorporation into hemoglobin and reflects functional availability of iron to the erythron. Thus, these new parameters can be useful in (1) measuring bone marrow engraftment following transplant, (2) determining bone marrow response to iron or erythropoietin therapy, (3) recognizing the potential cause for poor response to erythropoietin therapy, and (4) diagnosing iron-deficient states in infants and adolescents.[3–7]

Automated body fluid analysis is a feature that newer automated hematology instruments offer. Depending on the instrument, total nucleated cell (TNC) counts, leukocyte counts (WBC), erythrocyte counts (red blood cell [RBC]), and partial leukocyte differential counts are determined by the same technologies as whole-blood cell counts and leukocyte differentials. The potential advantages of automated body fluid analysis include improved turnaround time, increased precision, and decreased interoperator variability.[8] To achieve reliable body fluid cell counts, the automated hematology instruments must be able to enumerate cells at a much lower reportable range (e.g., WBC 0.0 $\times$ 10^3/mcL [μL]) compared to blood cell enumeration. Improvements to blood cell enumeration methods allow better

discrimination of cells, resulting in lower reportable ranges (linearity). Thus, body fluid analysis is now available on certain automated hematology instruments. The specific parameters and body fluids that can be evaluated are discussed with the instrument.

This chapter reviews the basic operating principles of several hematology instruments that are seen in the field. Other instruments use a combination of these principles.

☑ CHECKPOINT 39-1

What is the basis of the impedance principle for cell counting?

Impedance Instruments

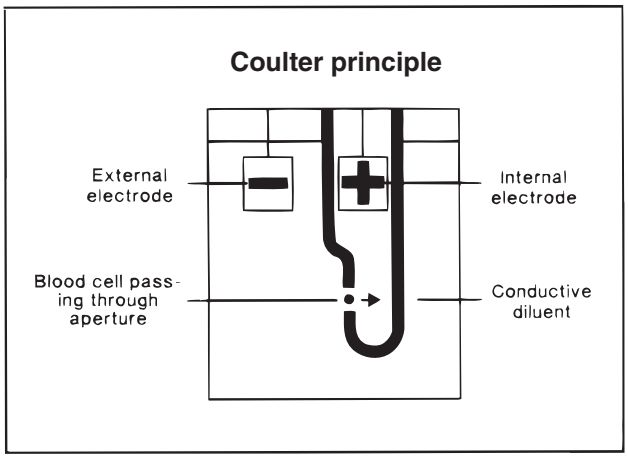
Of the seven instruments that use the impedance principle in cell counting, three are Beckman-Coulter instruments, three are Sysmex instruments, and one is an Abbott Diagnostics instrument. All also use some variance of optical light scattering for leukocyte differentials and reticulocyte enumeration.

Coulter® LH Series

The LH 750 (introduced in 2003) and the LH 780 (introduced in 2006) provide a CBC, five-part leukocyte differential, and reticulocyte count from a single blood sample aspiration. When the LH SlideMaker and LH SlideStainer are added to the workstation, Wright-stained peripheral smears are prepared and labeled from the same sample, decreasing the number of times the sample is handled and the potential for introducing random errors.

Instruments in the LH series use the basic principles of blood cell enumeration, reticulocyte enumeration, and determination of the five-part leukocyte differential that were hallmarks of earlier Coulter® models such as the Gen • S and STKS instruments.[9–15] The LH 750 and LH 780 provide several additional parameters that are advantageous for the differentiation of normal individuals from individuals with certain disease states. These parameters include the nucleated red blood cell (NRBC) count (as a percentage) and corrected WBC count that were introduced with the LH 750 instrument and the red cell distribution width-standard deviation (RDW-SD), which was introduced with the LH 780 instrument.

For these instruments, CBC analysis begins with the aspiration of the ethylenediaminetetraacetic acid (EDTA) anticoagulated blood sample by a closed tube system. The aspirated blood sample is divided into four aliquots. The first aliquot is delivered to the RBC/platelet dilution chamber where blood cells are diluted in an electrically conductive diluent. Within the dilution chamber is an external electrode and three **apertures**, each of which has an internal electrode (Figure 39-1 ■). As individual blood cells pass through an aperture, there is an increase in resistance (i.e., pulse height) between the external and internal electrodes that is proportional to the cell volume. A steady stream of diluent flows behind each aperture, preventing cells from reentering the aperture. **Threshold limits** are established for enumerating cells based on cell volume. Particles (cells) of more than

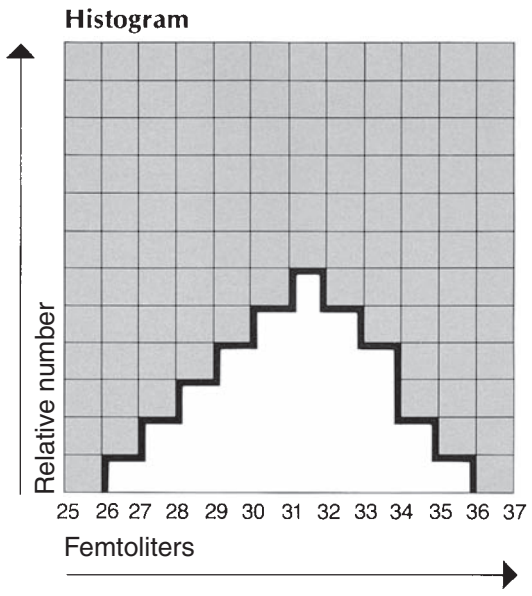


■ **FIGURE 39-1** Coulter principle. Increased electrical resistance, or impedance, occurs when the poorly conductive blood cell passes through the aperture.

Pierre R. *Seminar and Case Studies: The Automated Differential*. Hialeah, FL: Coulter Electronics, Inc.; 1985. Reprinted with permission

36 fL are counted as erythrocytes (RBCs). Because three apertures are located within the dilution chamber, three RBC counts are obtained. These counts are compared and evaluated by the instrument's data analysis system. If there is agreement, the reported RBC count represents the average of the counts from the three apertures.

An RBC **histogram** (size distribution curve) is created based on cell volume and relative cell number (Figure 39-2 ■). The RBC histogram allows the visualization of changes in size within the erythrocyte



■ **FIGURE 39-2** Histogram, or size distribution curve, allows the visualization of the blood cell population based on the relative cell number and its volume (in fL).

Significant Advances in Hematology. Hialeah, FL: Coulter Electronics, Inc.; 1983. Reprinted with permission.

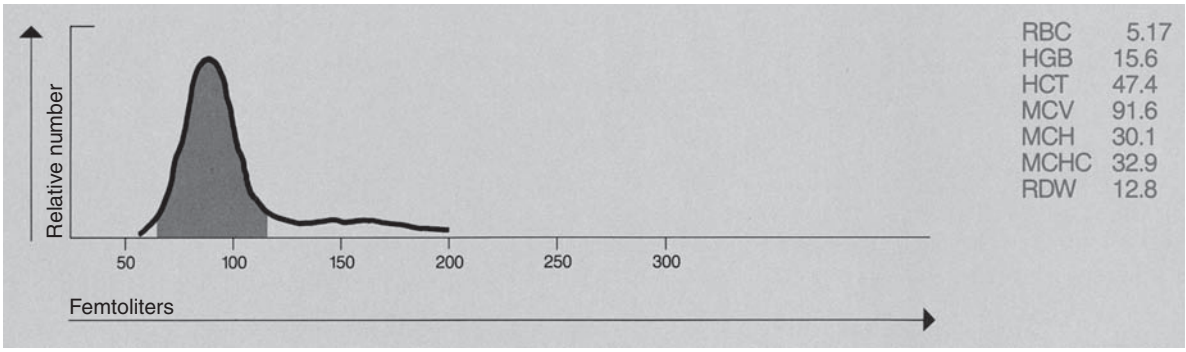


FIGURE 39-3 Red blood cell (RBC) histogram and count. The shaded area represents those cells used in the red cell distribution width (RDW) (i.e., RDW–coefficient of variation) calculation. The excluded cells can represent large platelets, platelet clumps, or electrical interference on the left and RBC doublets, RBC triplets, RBC agglutinates, or aperture artifacts on the right.

Significant Advances in Hematology. Hialeah, FL: Coulter Electronics, Inc.; 1983. Reprinted with permission.

population. For example, a shift to the left of the erythrocyte population correlates with microcytic erythrocytes, and the appearance of two separate peaks correlates with a dual population of erythrocytes (e.g., mixture of microcytic erythrocytes and normocytic erythrocytes). This information can be useful in diagnosing certain erythrocyte disorders.

The erythrocyte data obtained from the RBC/platelet dilution include the RBC count by direct measurement and mean cell volume (MCV), red cell distribution width (RDW)–coefficient of variation (CV) (i.e., RDW or RDW-CV), and RDW–standard deviation (RDW-SD), which are derived from the RBC histogram (Figure 39-3 ■). The RDW-SD is determined from the RBC histogram by calculating the width in femtoliters (fL) of the RBC population at the 20% height level on the histogram when the peak height is considered 100%[16] (Figure 39-4 ■). Both the RDW-CV and RDW-SD reflect the degree of anisocytosis within the erythrocyte population. The RDW-SD differs from the RDW-CV in that it directly measures the erythrocyte population; the RDW-CV bases its calculation on the population's standard deviation (SD) and MCV (RDW = 1 SD/MCV × 100%). Thus, a false RDW-CV can be observed in certain situations (Chapter 10). For example, if both the SD and MCV are elevated, the RDW-CV can appear normal when anisocytosis is actually present or if the SD is normal but the MCV is decreased, the RDW-CV can appear increased when in fact there is no anisocytosis. Because the MCV does not affect the RDW-SD, it is potentially a better indicator of the degree of anisocytosis within a given erythrocyte population. The RDW-SD can be used with other test results in differentiating iron deficiency anemia from heterozygous β thalassemia because a normal RDW-SD is more closely associated with heterozygous β thalassemia and an increased RDW-SD is associated with iron deficiency.[17] The RDW-SD is reported in fL; its reference interval is 39–47 fL in adults, whereas the RDW-CV's reference interval is 12.0–14.6%.

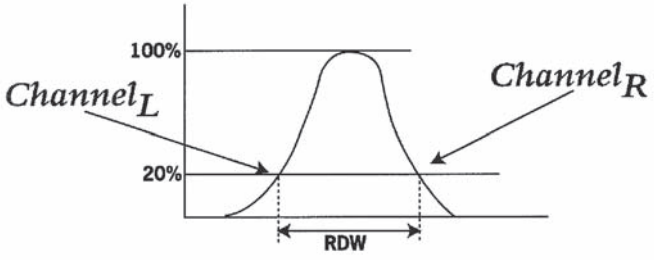


FIGURE 39-4 Calculation of red cell distribution width (RDW)–standard deviation (RDW-SD). The RDW-SD represents the width of the erythrocyte population at the 20% height level on the red blood cell histogram and is reported in fL. For example, if Channel$_L$ is 70 fL and Channel$_R$ is 111 fL, the RDW-SD is 41 fL (i.e., 111 − 70 = 41).

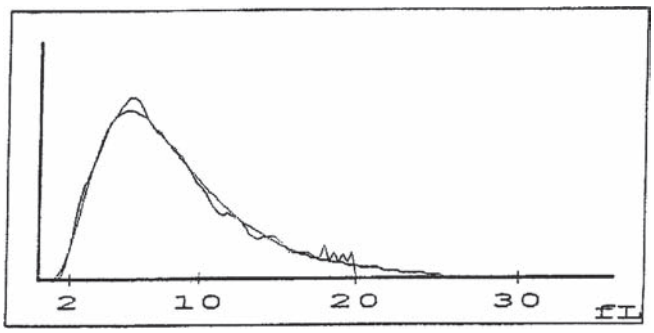
RDW-SD = red cell distribution width–standard deviation

Red Cell Distribution Parameters—(1) RDW-SD (2) RDW (CV), Technical Bulletin 9617. Brea, CA: Beckman-Coulter®; 2007. Reprinted with permission

The calculated RBC parameters are the hematocrit (Hct), which is calculated from the MCV and the RBC count (Hct = MCV × RBC); the mean cell hemoglobin (MCH), which is calculated from the RBC count and hemoglobin concentration; and the mean cell hemoglobin concentration (MCHC), which is calculated from the hemoglobin concentration and hematocrit (Chapters 10 and 37). The hemoglobin concentration is obtained from the WBC/hemoglobin dilution chamber.

The platelet count is also obtained from the RBC/platelet dilution chamber. Particles between 2 and 20 fL are counted as platelets, and a raw platelet histogram is obtained (Figure 39-5 ■). This histogram is evaluated to determine whether it is a log normal curve. The raw platelet histogram is electronically smoothed and extrapolated over 0–70 fL. The platelet count is derived from the extrapolated histogram. An additional parameter obtained from the platelet histogram is the **mean platelet volume (MPV)**, which is analogous to the erythrocyte MCV.

The second aliquot is delivered to the WBC/hemoglobin dilution chamber where the leukocyte (WBC) count and hemoglobin determinations are made. The WBC count is directly measured by electrical impedance from the leukocyte dilution after a lytic agent is added to the dilution. The lytic agent serves to lyse the erythrocytes, convert released hemoglobin to cyanmethemoglobin, and shrink the leukocyte cell membrane and cytoplasm. Therefore, the WBC count represents a measure of the cell volume rather than



■ **FIGURE 39-5** Normal platelet histogram. The jagged line represents the raw data collected from 2–20 fL. The smooth line represents the extrapolated histogram from 0–70 fL.

native cell size as it passes through the aperture. Particles larger than 35 fL are counted as leukocytes. Similar to the RBC count, WBC counts are obtained from three apertures in the WBC/hemoglobin dilution chamber, and the data analysis system compares and evaluates them. The reported WBC count represents an average of the three counts.

The WBC histogram represents the size distribution curve for the leukocyte data and allows visualization of subpopulations of cells based on their relative sizes. The WBC histogram is the basis of the three-part differential identifying lymphocytes as cells between 35 and 90 fL, mononuclear cells as cells between 90 and 160 fL, and granulocytes as cells between 160 and 450 fL (Figure 39-6 ■). The instrument's data analysis system examines the WBC histogram for the presence of interferences within one or more of the leukocyte populations and, using a system of region flags, alerts the laboratory professional to suspected abnormalities (Table 39-1 ★). The reason for a region flag can be found by carefully examining the peripheral blood smear. For example, presence of nucleated erythrocytes or clumped platelets is associated with a region 1 flag.

The hemoglobin concentration is determined by measuring the absorbance of cyanmethemoglobin in the WBC/hemoglobin chamber at 525 nm. Using Beer's law, this absorbance reading is proportional to the concentration of hemoglobin. Additionally, this instrument uses

☑ CHECKPOINT 39-2

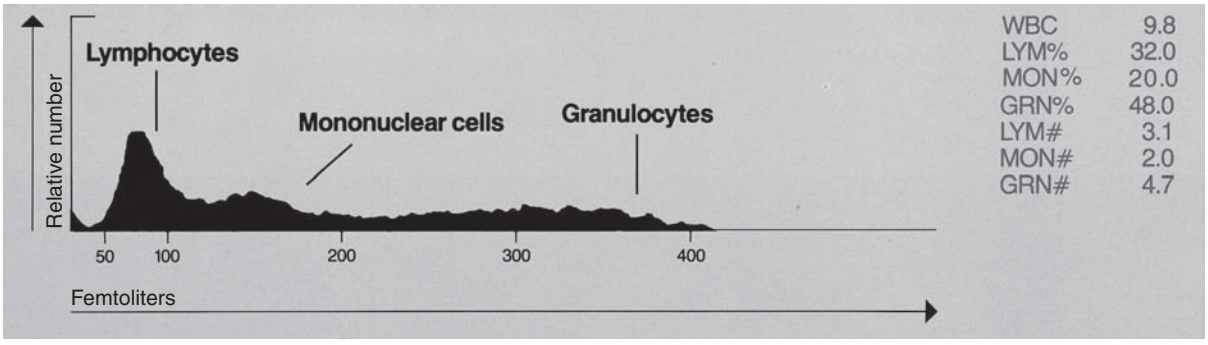
Why is lytic agent added to the leukocyte dilution?

a **reagent blank** at the beginning of each operating cycle to negate the effect of the reagent alone on the sample's absorbance reading.

A third sample aliquot is delivered to the orbital mixing chamber. Within this chamber, blood is mixed by gentle agitation with a heated lysing reagent to remove the erythrocytes while leaving the leukocytes in their near native state. A second stabilizing reagent is added to stop the lytic reaction and preserve the integrity of the leukocytes. This dilution is sent to the volume-conductivity-scatter (VCS) flow cell to determine the five-part leukocyte differential. The cells pass through the flow cell singly by **hydrodynamic focusing** (Chapter 40). As each cell passes through the flow cell, three separate measurements are taken simultaneously: cell volume, cell conductivity, and cell's light scatter characteristics. Impedance determines cell volume; high-frequency electromagnetic probe determines cell conductivity, which evaluates internal physical and chemical constituents; and a helium-neon laser determines light scatter characteristics such as cell surface, shape, and reflectivity. The instrument's internal system monitors and adjusts reaction characteristics and the data analysis of the VCS-derived characteristics to differentiate the leukocyte cell types.

The Intellikinetics™ application monitors and reacts to changes in the external environment (e.g., changes in ambient room temperature). With this application, the LH instruments are able to maintain consistent reaction conditions and eliminate cellular analysis problems because of inconsistent location of cells within three-dimensional space. Data analysis is achieved through the use of the AccuGate™ algorithm. The different leukocytes within a sample are identified and classified by **contour gating** that is individualized for each sample (Figure 39-7 ■). The advantage of contour gating over traditional linear gating is its ability to differentiate overlapping cell populations (e.g., monocytes from reactive lymphocytes).

If NRBCs are detected during the analysis of a CBC and differential, the number of NRBCs (i.e., NRBC%) is determined. Enumeration of NRBCs begins with their detection on the WBC scatterplot



■ **FIGURE 39-6** White blood cell (WBC) histogram and count. In a normal patient, the lymphocyte region represents lymphocytes, the mononuclear region represents monocytes, and the granulocyte region represents neutrophils, eosinophils, and basophils.

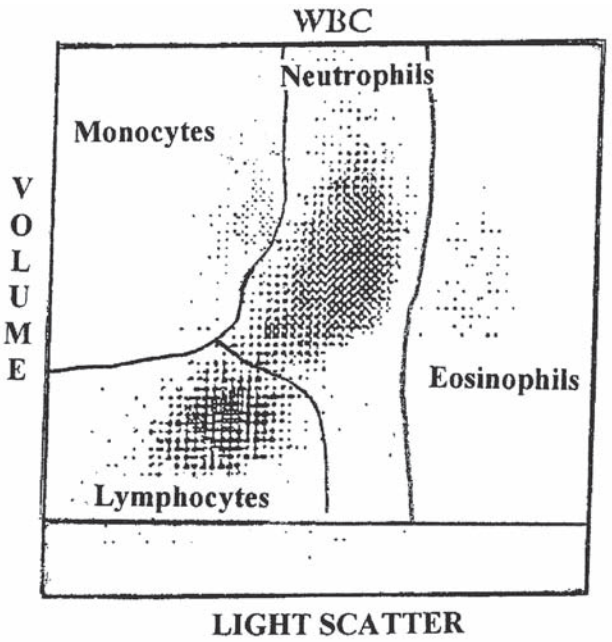
Significant Advances in Hematology. Hialeah, FL: Coulter Electronics, Inc.; 1983. Reprinted with permission.

★ **TABLE 39-1** Coulter® WBC Histogram Region Flags

Region Flag	Affected Position	Possible Abnormalities
R1	Lymphocyte population does not begin at baseline (~35 fL)	Nucleated erythrocytes, large platelets, clumped platelets, or intracellular parasites (e.g., malaria)
R2	No valley between lymphocyte and mononuclear populations (~90 fL)	Reactive lymphocytes, blast cells
R3	No valley between mononuclear and granulocyte populations (~160 fL)	Eosinophilia, basophilia, presence of immature neutrophils
R4	Granulocyte population does not return to baseline (~450 fL)	Granulocytosis
RM	Interference detected in multiple positions	

Other impedance-based instruments providing three-part leukocyte differentials use a flagging system similar to the Coulter® instruments.

generated by VCS technology[18] (Figure 39-8 ■). If nucleated red blood cells are suspected based on their characteristic signature, the computer system examines the WBC histogram generated by impedance for the presence of cells to the left of the lymphocyte population. If both plots indicate that NRBCs could be present, the computer system evaluates the combined information to rule out giant platelets or small lymphocytes. If all data analyses point to the presence of NRBCs, the number of NRBCs per 100 leukocytes (NRBC%) is derived from data collected by VCS technology and the application of an algorithm to the WBC count. The absolute NRBC count (NRBC#) represents the total number of nucleated red blood cells in the blood sample and is calculated from the NRBC% and the total WBC count (i.e., NRBC# = NRBC% × WBC count). Because increased numbers of NRBCs falsely increase the total WBC count, it is corrected for their presence, and the corrected WBC count is reported.



■ **FIGURE 39-7** White blood cell (WBC) scatterplot.WBC scatterplot graphs volume versus light scatter and reveals the locations of four leukocyte populations in two-dimensional space. The basophil population is located behind the lymphocytes but in three-dimensional space is clearly delineated.

In the heated reticulocyte dilution chamber, the fourth aliquot is mixed with new methylene blue reagent. The residual RNA is precipitated within the reticulocytes. The stained sample is then mixed with an acidic, hypotonic solution that (1) elutes hemoglobin from the erythrocytes but permits precipitated RNA to remain and (2) spheres the erythrocytes. This sample is then sent to the VCS flow cell for analysis. The light scatter data generated by flow cytometric examination of sphered erythrocytes and reticulocytes are more reproducible than are data generated from nonsphered erythrocytes and reticulocytes. The sphering of mature erythrocytes and reticulocytes eliminates the error inherent in flow cytometric examination of irregularly shaped erythrocytes. Contour gating of the VCS-derived characteristics is used to classify reticulocytes versus mature erythrocytes and to identify the immature reticulocyte fraction (Web Figure 39-1; Chapter 11). The reticulocyte parameters obtained by this analysis include absolute reticulocyte count, reticulocyte percentage, immature reticulocyte fraction, and mean reticulocyte volume (i.e., MCVr).

The computer system compiles all data obtained from the instrument's analysis. For the cell counts, the instrument's computer corrects the counts for **coincidence** (two or more cells passing through the aperture at the same time) and compares the counts for replication (e.g., compares the triplicate RBC counts). If two or three of the counts do not agree, the count (i.e., RBC count) is not reported but recorded as "vote-out." If there is agreement, the reported count represents the average of the counts. The instrument's computer also evaluates the data to detect abnormalities and determines the reported parameters (see Web Table 39-1 for the reported parameters). The reported results and selected histograms/**scatterplots** can be displayed on the computer screen, printed to a hard copy (Figure 39-9 ■), or transferred to the laboratory information system (LIS). If abnormalities are detected, suspect flags (software-generated flags) or user-defined flags (definitive flags) alert the laboratory professional who uses this information to correlate CBC data with peripheral blood morphology to improve the identification and confirmation of abnormalities.

☑ CHECKPOINT 39-3

Explain how the Coulter® LH 780 determines the five-part leukocyte differential.

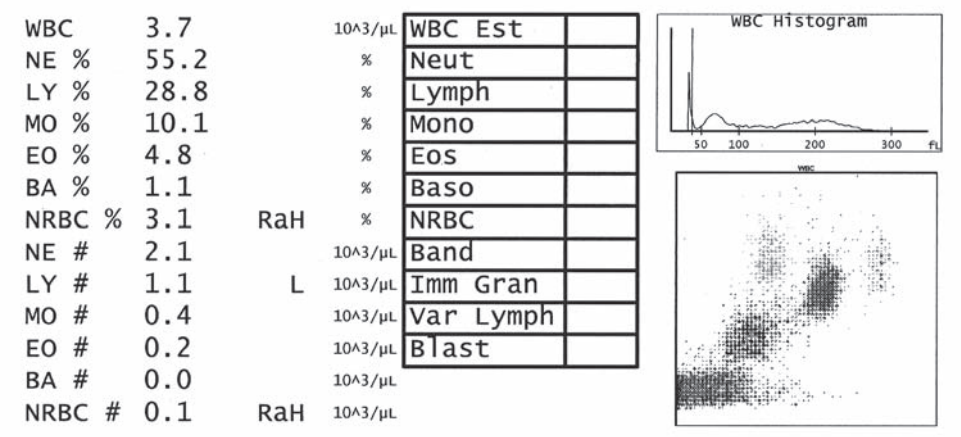


FIGURE 39-8 Coulter® LH 750 white blood cell (WBC) scatterplot and WBC histogram depiction of the presence of nucleated erythrocytes. On the WBC scatterplot, detection of cells between the lymphocyte population and red blood cell ghosts suggests nucleated erythrocytes, which are suspected if there is a population of cells to the left of the lymphocyte population on the WBC histogram. The nucleated red blood cell percentage is determined if both observations are made and other potential interferences are ruled out (e.g., giant platelets).

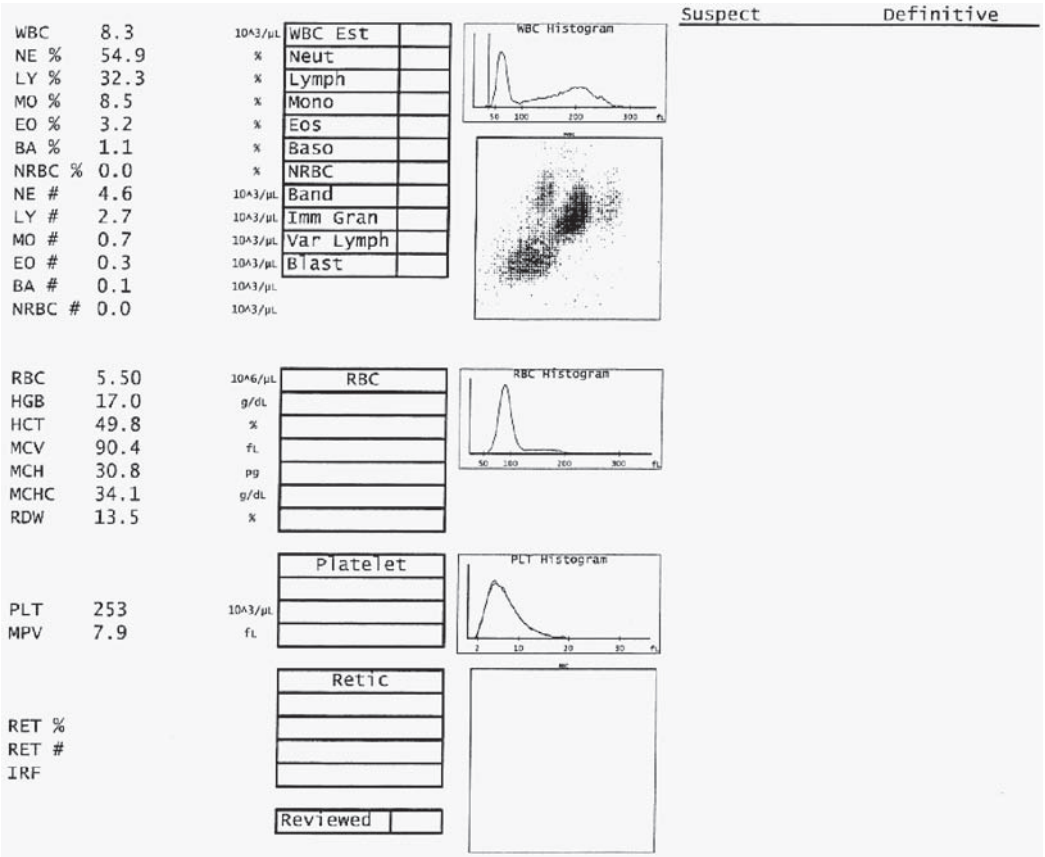
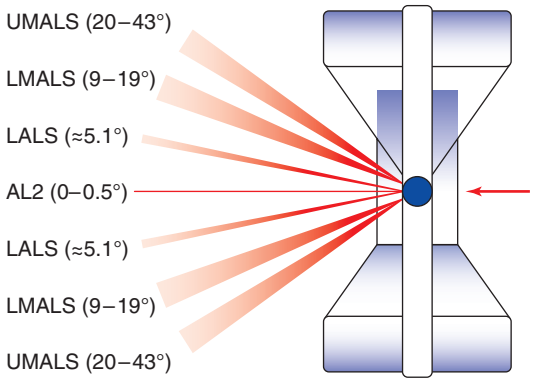


FIGURE 39-9 Coulter® LH 750 report from a normal individual.

Beckman-Coulter Unicel® DxH 800

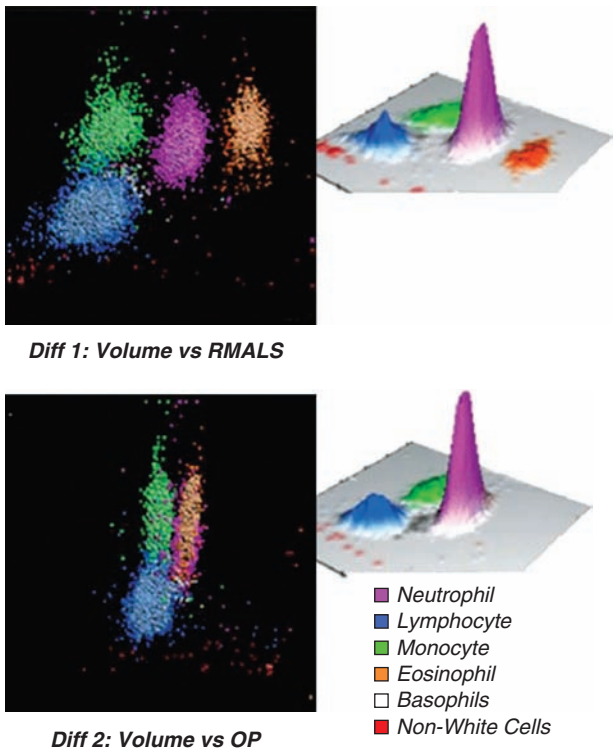
The Unicel® DxH 800 instrument determines the CBC using the same impedance methods as discussed with the Coulter® LH series instruments.[19] The system manager processes the data obtained from these measurements to generate the CBC parameters and WBC, RBC, and platelet histograms. Improvements have been made in the leukocyte differential, nucleated red blood cell determination, and reticulocyte enumeration. All three determinations occur in the VCSn module (next generation of VCS technology). The blood sample is delivered by the sample aspiration module (SAM) and dispensed directly to the specific mix chamber (e.g., Diff mix chamber). The appropriate temperature-controlled reagents (e.g., Erythrolyse and Stabilyse for Diff) are added to the mix chamber where sample and reagent mixing occurs. Following sample preparation, the prepared sample is delivered to the multitransducer module (MTM) for flow cell analysis. For the WBC differential, the DxH 800 instrument acquires data by evaluating each leukocyte in its near native state using three independent assessments: cell volume, cell conductivity, and cell light scatter. Cell volume and cell conductivity (i.e., radiofrequency opacity) are determined by the same methods as the Coulter® LH series uses. The difference is in the detection of light scatter. Within the MTM, multiple sensors are arranged around the flow cell to collect three measures of light scatter: lower median angle light scatter (LMALS), upper median angle light scatter (UMALS), and low-angle light scatter (LALS)[19–21] (Figure 39-10 ■). An additional sensor located in the laser path measures axial light loss (AL2), that is, the amount of light removed because of absorption and light scatter. The fifth light scatter measure, median angle light scatter (MALS), is the sum of the UMALS and LMALS. MALS, LMALS, and UMALS detect the cell's granularity and membrane topography. AL2 evaluates the cellular transparency, and LALS is used as a cellular complexity index.[20,21] The system manager evaluates raw data from the flow cell analysis and applies an advanced algorithm to the data to separate and identify the leukocyte populations, as well as common cellular interferences (e.g., large platelets), and calculates the frequency of cells within each population. Two 2-dimensional dataplots that show the five leukocyte populations—lymphocytes (LYs), monocytes (MOs), neutrophils (NEs), eosinophils (EOs), and basophils (BAs)—are generated. The Diff 1 dataplot is created by plotting volume on the y-axis and rotated median angle light scatter (RMALS) on the x-axis and clearly reveals the five leukocyte populations. The Diff 2 dataplot is created by plotting volume on the y-axis and opacity (OP) on the x-axis (Figure 39-11 ■). A 3-dimensional dataplot is also generated that classifies cells by volume, RMALS, and opacity. The system manager evaluates the data collected by the VCSn module to determine the percentage of each leukocyte (e.g., lymphocyte). The absolute count for each leukocyte is calculated based on its percentage and WBC count.

NRBCs are enumerated in the VCSn module. In the NRBC mix chamber, the blood sample is diluted with DxH Diluent and DxH Cell Lyse is added. The lyse reagent removes the non-nucleated red blood cells but keeps the NRBCs, leukocytes, platelets, and any cellular debris intact. Using flow cell analysis, each cell is evaluated for the characteristics of volume, conductivity, and light scatter. The specific light scatter measurements used to determine NRBCs are AL2 and LALS.[19,20] The AL2 measurement observed with the nucleus of an NRBC is clearly different from that observed with a leukocyte (e.g., lymphocyte). The system manager applies a dedicated algorithm to these raw data



■ **FIGURE 39-10** Light scatter measurements from the Unicel® DxH 800 flow cell. The upper median angle light scatter (UMALS) and lower median angle light scatter (LMALS) detect the cell's granularity and membrane topography. The axial light loss (AL2) evaluates cellular transparency and the low-angle light scatter (LALS) is used as a cellular complexity index.

Advancements in Technology: WBC Differential Methodology, Technical Bulletin 9403. Brea, CA: Beckman-Coulter®; 2009, Reprinted with permission



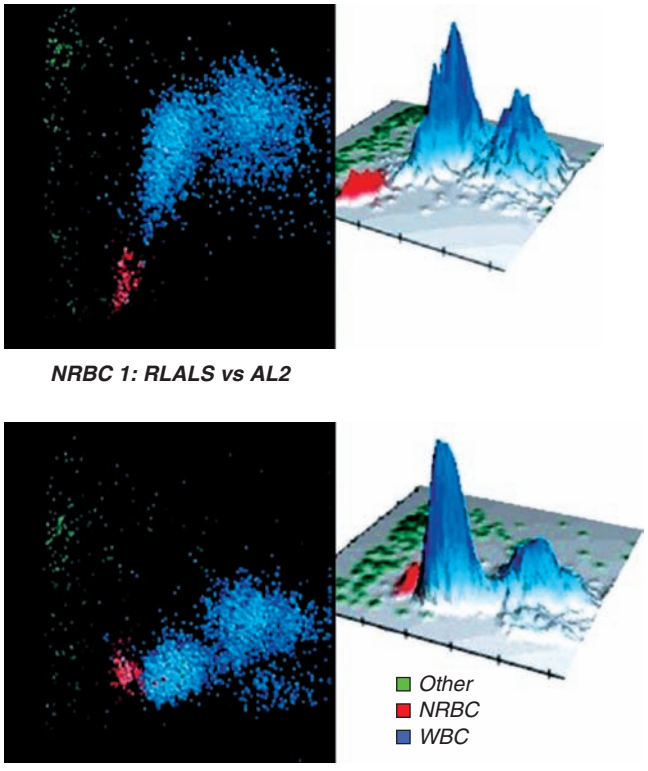
■ **FIGURE 39-11** Unicel® DxH 800 white blood cell differential dataplots. Diff 1 dataplot graphs volume versus rotated median angle light scatter (RMALS) and Diff 2 dataplot graphs volume versus opacity (OP). These dataplots reveal five leukocyte populations in two-dimensional space.

Advancements in Technology: WBC Differential Methodology, Technical Bulletin 9403. Brea, CA: Beckman-Coulter®; 2009, Reprinted with permission

to detect and isolate NRBCs from leukocytes, platelets, and cellular debris. Two 2-dimensional dataplots are created: the NRBC1 and the NRBC2. The NRBC1 dataplot depicts rotated low-angle light scatter (RLALS) on the y-axis and AL2 on the x-axis, whereas NRBC2 dataplot depicts rotated upper median angle light scatter (RUMALS) on the y-axis and AL2 on the *x*-axis (Figure 39-12 ■). The NRBC% is determined from the data collected by the VCSn module and analyzed by the system manager. The NRBC# is calculated. Because NRBCs interfere with the WBC enumeration by impedance, the WBC is corrected for their presence. In addition, if large platelets or platelet clumps are detected during this evaluation, the appropriate flag alerts the laboratory professional.

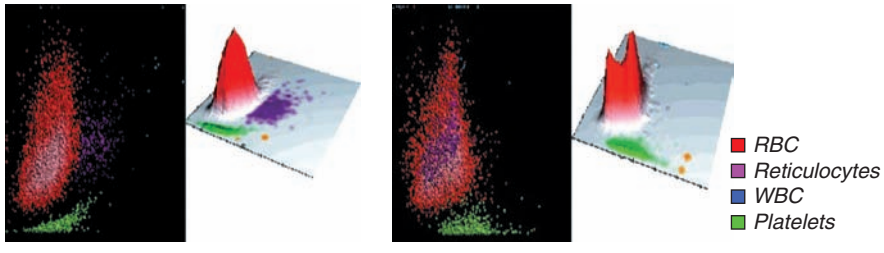
In the reticulocyte mix chamber, the blood sample is mixed with new methylene blue, which precipitates the residual RNA in the reticulocytes. Then, reticulocyte-clearing reagent is added. This reagent stabilizes the dye-reticulum complex and spheres the erythrocytes and reticulocytes, similar to the method the Coulter® LH series uses. The MTM evaluates each cell for cell volume, cell conductivity, and five light scatter characteristics. Using these data, the system manager applies an advanced algorithm to discriminate reticulocytes from mature erythrocytes. The system manager generates 2-dimensional dataplots (RETIC1 and RETIC2) that reveal mature red cells and reticulocyte populations. The RETIC1 dataplot depicts cell volume (*y*-axis) versus linear light scatter (LLS) on the *x*-axis, whereas the RETIC2 dataplot depicts cell volume (*y*-axis) versus OP on the *x*-axis (Figure 39-13 ■). The reticulocyte percentage (RETIC%) is determined from the data collected in the VCSn module and analyzed by the system manager. The calculated parameters include absolute reticulocyte count (RETIC% × RBC count), IRF, and mean reticulocyte volume (MRV).

The system manager evaluates all data collected for the CBC, leukocyte differential, and NRBC determinations, including the histograms and dataplots that are generated, and determines whether an interference is present. The CBC parameters are corrected for those interferences that are clearly identified. For example, the WBC is corrected for the presence of NRBCs but the WBC histogram will not be flagged. On the other hand, if the WBC is elevated ($>11 \times 10^3$/mcL), the hemoglobin will be corrected and if the WBC exceeds 140×10^3/mcL, the RBC will also be corrected.[22] The reported results and selected histograms/scatterplots can be displayed on the computer screen, printed to a hard copy, or transferred to the laboratory information system (LIS) (Web Table 39-1). If abnormalities are detected, the DxH 800 uses suspect, system, and definitive flags to alert the laboratory professional. New flagging algorithms applied during data analysis decrease the number of false flags, minimizing



■ **FIGURE 39-12** Unicel® DxH 800 nucleated red blood cell (NRBC) dataplots. NRBC 1 dataplot graphs rotated low-angle light scatter (RLALS) versus axial light loss (AL2) and NRBC 2 dataplot graphs rotated upper median angle light scatter (RUMALS) versus AL2. These dataplots clearly delineate the nucleated erythrocyte population from the leukocyte population.

Advancements in Technology: NRBC Enumeration, Technical Bulletin 9404. Brea, CA: Beckman-Coulter®; 2009, Reprinted with permission



■ **FIGURE 39-13** Unicel® DxH 800 Reticulocyte dataplots. RETIC 1 dataplot graphs volume versus linear light scatter (LLS) and RETIC 2 dataplot graphs volume versus opacity (OP). These dataplots provide delineation of reticulocytes from mature erythrocytes and platelets.

Advancements in Technology: Reticulocyte Methodology, Technical Bulletin 9405. Brea, CA: Beckman-Coulter®; 2009, Reprinted with permission

the need for peripheral blood smear reviews and decreasing the turnaround times.

The Unicel® DxH 800 instrument generates erythrocyte counts (RBC) and total nucleated cell (TNC) counts on body fluid specimens from data obtained by its CBC module (i.e., impedance methodology).[19] Any body fluid (e.g., cerebrospinal fluid [CSF], serous, or synovial) is acceptable for analysis. However, synovial fluids should be pretreated with hyaluronidase to reduce the sample's **viscosity** (Chapter 30). In addition, running diluent as a body fluid sample immediately before analysis of a body fluid specimen is recommended to check the background counts of RBCs and TNCs. Each laboratory should establish criteria for acceptable background counts when performing body fluid analysis.

☑ CHECKPOINT 39-4

The following results were obtained from a blood sample analyzed by the Unicel® DxH 800: NRBC# = 2.7 × 10^3/mcL and corrected WBC = 18.1 × 10^3/mcL. What was the uncorrected WBC count?

Sysmex XE-Series™

The XE-2100™ instrument (introduced in 1999) and XE-5000™ instrument (introduced in 2007) use four different technologies including impedance (i.e., direct current), radio frequency, absorption spectrophotometry, and flow cytometry with fluorescent dyes to determine CBC, five-part leukocyte differential, nucleated erythrocytes, and reticulocyte count.[23–26]

Hemoglobin is measured in a designated channel using the sodium lauryl sulfate (SLS) method. A portion of the aspirated blood sample is first diluted with reagent diluent and then SLS-hemoglobin reagent is added. The surfactants within this reagent lyse the erythrocytes and release hemoglobin. SLS converts ferrous iron to ferric iron, forming methemoglobin. Methemoglobin combines with SLS to form the SLS-hemichrome molecule. The absorbance of this molecule is measured at 555 nm to determine the hemoglobin concentration (grams per deciliter) using Beer's law. This method is advantageous for two reasons: (1) free hemoglobin is rapidly converted to a detectable chromogen, decreasing measurement time, and (2) the reagent is cyanide free.

The erythrocytes and platelets are enumerated using impedance with hydrodynamic focusing. The blood sample is diluted with isotonic diluent and sent to the RBC/PLT sample chamber. A sheath fluid injector piston delivers the diluted blood sample to the direct current (DC) detection block. The blood cells flow through the aperture, and resistance (i.e., pulse height) is detected between the internal and external electrodes. The hydrodynamic focusing created by the sheath fluid aligns the blood cells to enter the center of the aperture singly, thus eliminating abnormal cell pulses generated when cells touch the sides of the aperture as they pass through. At the rear of the aperture, the sweeping flow of the back sheath fluid prevents blood cells from drifting back and generating false pulses. The RBC count and platelet count are directly measured; RBC and platelet histograms are generated; and the Hct is calculated from the pulse heights of the individual erythrocytes within the erythrocyte population. The pulse height of an individual erythrocyte is directly proportional to its volume.

The RBC and platelet counts are determined from the generated data by using automatic discriminators, similar to threshold limits used by the Coulter® instruments. The RBC count represents the number of cells that fall between a lower discriminator (25–75 fL) and an upper discriminator (200–250 fL). The data analysis system examines the raw data collected for a given blood sample's RBC count and then sets the specific lower and upper discriminator for determination of the reported RBC count. Likewise, the platelet count represents the number of cells that fall between a lower discriminator (2–6 fL) and an upper discriminator (12–30 fL). A fixed discriminator at 12 fL is also included in determining the platelet count to evaluate whether potential interferences exist that can result in an erroneous platelet count (e.g., presence of erythrocyte fragments). This platelet count is compared to the platelet count determined from the reticulocyte (RET) channel.

Other parameters derived or calculated based on data from the RBC/platelet dilution include MCV, MCH, MCHC, RDW-SD, RDW-CV, and MPV. The RDW-SD and RDW-CV are determined in the same manner as described for the Coulter® LH 780 instrument.

Two separate channels (i.e., DIFF and WBC/BASO) are used to generate the total leukocyte (WBC) count and the five-part leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). In the DIFF channel, a portion of the aspirated blood sample is diluted with lyse reagent. The lyse reagent removes the erythrocytes and creates "holes" in the cytoplasmic membrane of nucleated cells allowing a fluorescent dye (i.e., polymethine) to enter the cell and bind to cellular DNA and RNA. This dilution is sent to the optical detection block within which the cells pass singly through a laser beam (λ = 633 nm) emitted by the semiconductor diode laser. Each cell generates forward scatter (reflects cell size), side scatter (reflects cell complexity), and fluorescent intensity (reflects amount of bound fluorescent dye), depending on the cell type. The DIFF scattergram represents a plot of side scatter versus fluorescent intensity. The instrument's adaptive cluster analysis system (ACAS) allows clear separation of the cell populations depicted on the DIFF scattergram, including RBC ghosts, lymphocytes, monocytes, eosinophils, and neutrophils + basophils (Figure 39-14 ■). By applying the immature granulocyte (IG) master software to the data generated in the DIFF channel, the IG population (IG count) is enumerated (Web Figure 39-2). The IG count includes metamyelocytes, myelocytes, and promyelocytes. Studies indicate that the automated IG count is an acceptable replacement for the manual morphology count of IGs, but studies regarding the usefulness of the IG count to screen for sepsis and infection are conflicting.[27,28] Further studies are needed to clarify its use.

The blood sample is diluted, and a special lyse reagent that lyses erythrocytes and shears all leukocytes except basophils is added in the WBC/BASO channel. The optical detection block analyzes the dilution to capture each cell's forward and side scatter as it passes through the laser beam. The adaptive cluster analysis system evaluates this information to determine a total leukocyte (WBC) count and the basophil count (i.e., basophil percentage and absolute basophil count). The WBC/BASO scattergram (Web Figure 39-3) is a plot of

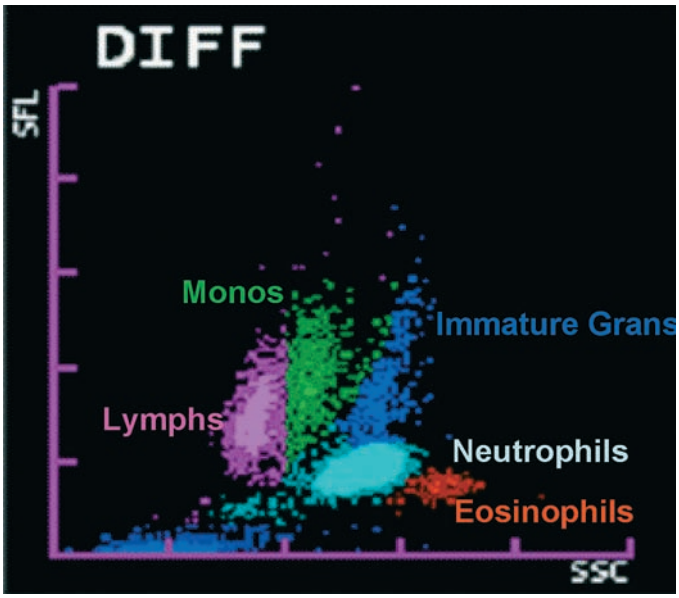


FIGURE 39-14 DIFF scattergram, Sysmex XE-2100™. RBC ghosts, lymphocytes, monocytes, eosinophils, and nutrophils + basophils are identified. Basophils are specifically identified in the WBC/BASO scattergram.
Courtesy of Sysmex Corporation, Kobe, Japan.

side scatter versus forward scatter. On this scattergram, the basophils are easily distinguished from the other leukocytes and RBC ghosts because of their size and complexity (i.e., high forward scatter and high side scatter).

In the immature myeloid information (IMI) channel, reagents act selectively on the lipid membranes of the leukocytes. Mature leukocytes with phospholipid-rich membranes are completely lysed, leaving only bare nuclei. The immature myeloid cells remain intact. In the IMI channel, the cells are analyzed by direct current and radio frequency (reflecting a cell's internal structure and density) to determine the degree of immaturity (Web Figure 39-4). This channel allows a clear delineation of immature granulocytes and blasts. With the HPC master software, this channel provides the hematopoietic progenitor cell (HPC) count. Studies have shown that the HPC count correlates well with the CD34+ cell count by flow cytometry. Thus, HPC counts can be used to determine peripheral blood stem cell counts to assess optimal harvesting time following peripheral blood stem cell mobilization[29] (Chapter 29).

Differentiation of similar cell types is also achieved on the XE-5000™ instrument with its efficient multichannel messaging (eMM) software and algorithms that improve the detection of blasts, abnormal lymphocytes/lymphoblasts, and atypical lymphocytes. For example, application of these algorithms triggers the blast flag when the software detects cells in the blast areas of the DIFF scattergram and of the IMI scattergram. The atypical lymphocyte flag occurs when cells are detected in the atypical lymphocyte area of the DIFF scattergram, but no cells are detected in either the abnormal lymphocyte/lymphoblast area or the blast area of the IMI scattergram. This advanced flagging system has the potential to decrease the number of peripheral blood smear reviews.[30]

☑ CHECKPOINT 39-5

How does the determination of the five-part leukocyte differential for the Unicel® DxH 800 instrument and the Sysmex XE-Series™ instrument differ?

Nucleated erythrocytes are enumerated in the NRBC channel. Within this channel, the blood cells are diluted with a lyse plus fluorescent dye reagent. This reagent hemolyzes the erythrocytes, removes the nucleated erythrocytes' cytoplasmic membrane, and shrinks the nucleus. The fluorescent dye binds to the nuclei. The reagent only perforates the cytoplasmic membrane of the leukocytes, allowing fluorescent dye to enter the cell and bind to intracytoplasmic organelles and the nucleus, but does not alter the leukocytes' shape. Thus, leukocytes exhibit stronger fluorescence than nucleated erythrocytes. As the nuclei of the nucleated erythrocytes and the intact leukocytes pass through the laser beam of the optical detection block, they generate forward scatter, side scatter, and fluorescence. This information is used to create the NRBC scattergram, which plots fluorescent intensity versus forward scatter (Figure 39-15 ■). Leukocytes with their high fluorescent intensity and high forward scatter (i.e., larger size) are clearly delineated from the nucleated erythrocytes. This analysis provides a sensitive method to determine nucleated erythrocytes as low as 0.1 NRBC/100 WBCs. The instrument's data analysis system uses the nucleated erythrocyte count (NRBC#) to correct the total leukocyte (WBC) count and lymphocyte count for their presence. The lymphocyte count is affected because nucleated erythrocytes and lymphocytes fall in the same cluster on the DIFF scattergram.

The reticulocyte count is determined in the RET channel. Within this channel, the fluorescent dye containing oxazine and polymethine penetrates the cell membranes and stains the blood cells. Oxazine

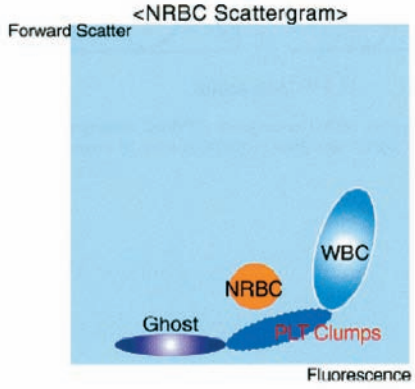


FIGURE 39-15 Nucleated red blood cell (NRBC) scattergram, Sysmex XE-2100™. Nucleated erythrocytes are clearly delineated from leukocytes based on their lower fluorescence intensity and smaller size (i.e., less forward scatter) as seen on this schematic pattern. WBC, white blood cell; PLT, platelet
Rowan RM, Linssen J. *A picture is worth a thousand words. Sysmex Journal International.* 2005;15(1):27–38. Reprinted with permission.

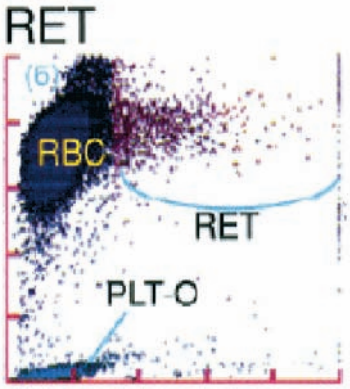
binds to residual RNA within the reticulocytes, and polymethine binds to RNA and DNA within nucleated cells. The diode laser of the optical detection block evaluates each blood cell and determines its forward scatter and fluorescence. The data analysis system uses this forward scatter and fluorescence information to generate the RET scattergram. From this scattergram, cells are classified as mature erythrocytes, reticulocytes, and platelets (Figure 39-16 ■). The reticulocytes are further classified based on fluorescent activity to determine reticulocyte maturity (Web Figure 39-5). Reported parameters include absolute reticulocyte count, reticulocyte percentage, low fluorescence ratio (LFR), middle fluorescence ratio (MFR), high fluorescence ratio (HFR), IRF, reticulocyte hemoglobin content (Ret He), and platelet count. The IRF represents the MFR and HFR combined and is reported as a percentage of the total reticulocyte count.

The platelet count from the RET channel is designated the PLT-O (platelet-optical) count and is compared to the platelet count from the RBC/platelet dilution. If the platelet count is extremely low, the count from the RET channel is the reported platelet count because it is more accurate as the result of the additional cellular characteristics that are evaluated. The immature platelet fraction (IPF) is an additional platelet parameter derived from the RET channel that represents the **reticulated platelets**, which are newly released platelets that possess residual RNA and exhibit fluorescence because the RNA binds oxazine. An increased IPF reflects increased thrombopoiesis, whereas a decreased IPF reflects decreased platelet production. The IPF is useful in a variety of clinical situations (1) as an indicator of platelet engraftment following stem cell transplantation, (2) in the diagnosis of thrombocytopenia associated with increased platelet destruction versus bone marrow failure, and (3) as a requirement of platelet transfusion associated with cytotoxic therapy or stem cell transplant.[31–34]

The XE-5000™ instrument determines several additional erythrocyte parameters. Because the instrument measures the hemoglobin content of individual erythrocytes (i.e., mature erythrocytes, reticulocytes) during the reticulocyte enumeration (within RET channel), a new software package allows the data management system to calculate the percentage of hypochromic erythrocytes (%HYPO HE) and hyperchromic erythrocytes (%HYPER HE). Using the pulse height (i.e., volume) for each erythrocyte counted, the data management system generates the percentage of microcytes (%MICRO R) and percentage of macrocytes (%MACRO R).[30,35] Studies indicate that the new parameters, %HYPO HE and %MICRO R, have potential use in the differentiation of the β-thalassemia trait from other microcytic anemias and that %HYPO HE may be a useful parameter in the evaluation of iron status.[35–37]

The data analysis system analyzes all data, including cell counts, histograms, and scattergrams. The results are displayed on the computer screen, printed to a hard copy (Figure 39-17 ■), or transferred to the LIS (see Web Table 39-1 for reported parameters). An extensive flagging program with interpretive comments alerts the laboratory professional to abnormal results. Use of the flagging system and the observations of the scattergrams and histograms allow the laboratory professional to focus on specific abnormalities when performing a peripheral blood smear evaluation.

The body fluid mode of the XE-5000™ performs erythrocyte counts (RBC), leukocyte counts (WBC), two-part differentials (polymorphonuclear cells and mononuclear cells), and total counts (TC-BF) on all body fluids (e.g., cerebrospinal, serous, synovial fluids).[38–40] No pretreatment of the sample is required. Using the body fluid mode, the cell-counting time is extended to increase the precision of the cell counts. The RBC count is determined by impedance, and the WBC count and two-part differential are determined by fluorescent flow cytometry. The data collected in these measurements are analyzed by specific algorithms to generate the cell counts and two-part differential. When the laboratory professional switches the instrument from the whole blood mode to the body fluid mode, the instrument performs a rinse cycle to prevent carryover from previously tested whole blood samples as well as a background check before the body fluid sample is tested.



■ **FIGURE 39-16** Reticulocyte (RET) scattergram, Sysmex XE-2100™. Mature erythrocytes, RETs, and platelets (PLTs) are identified.

RBC = red blood cell

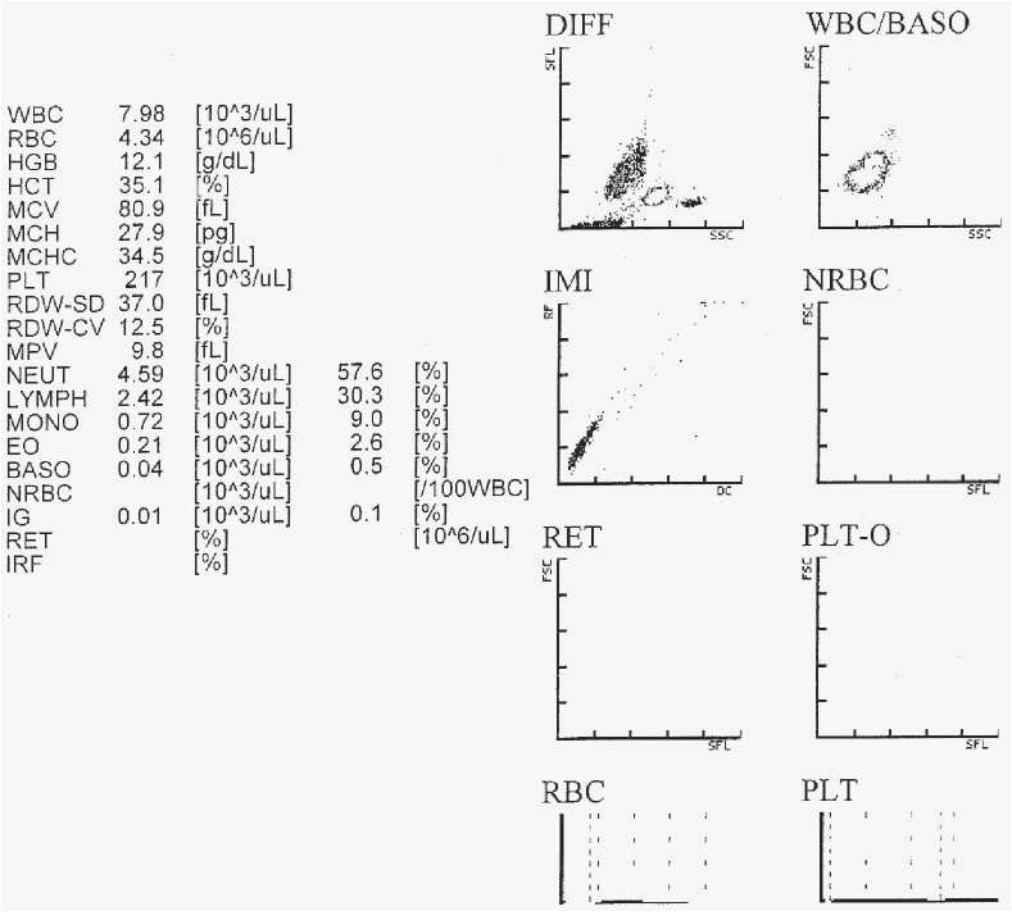
Rowan RM, Linssen J. *A picture is worth a thousand words. Sysmex Journal International*. 2005;15(1): 27–38. Reprinted with permission.

Sysmex XN-Series™

Introduced in 2012, the XN-10™ instrument represents the latest in Sysmex hematology instrumentation. The XN-10™ instrument is a modular component capable of determining the CBC, six-part leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils, basophils, and IGs), nucleated erythrocyte count, and reticulocyte count from a single aspiration of a blood sample. (See Web Table 39-1 for reported parameters.) The addition of the SP-10™ slidemaker/stainer creates a complete modular workstation. To increase the productivity of a workstation, additional XN-10™ instruments can be added. For example, the XN-3000™ is a workstation with two XN-10™ modular units and the SP-10™ slidemaker/stainer unit.

The XN-series™ instrument uses the same methods as described for the XE-series™ for determining the erythrocyte (RBC) count, hemoglobin, RBC parameters (e.g., Hct, MCV), platelet count (impedance), reticulocyte count, and reticulocyte parameters (e.g., IRF, Ret He). New methods allow clearer discrimination of leukocytes, abnormal leukocytes, and nucleated erythrocytes.[41,42]

The white cell differential (WDF) channel differentiates and enumerates neutrophils and basophils, lymphocytes, monocytes, eosinophils, and IGs (Figure 39-18 ■). These cells are classified based on their side scatter and fluorescent intensity, characteristics that are similar to those used for cell classification by the DIFF channel of the XE-series™



■ **FIGURE 39-17** Sysmex XE-Series™ report from a normal individual.

instrument. The key difference is the instrument's new algorithm, Sysmex Adaptive Flagging Algorithm, based on Shape recognition (SAFLAS) that allows better discrimination of monocytes, lymphocytes, atypical lymphocytes, and blasts based on analysis of the differences in these cell clusters. Application of this algorithm improves the specificity of alert flags for atypical lymphocytes and blasts.

In addition, accurate leukocyte differentials are obtained for blood samples with leukocyte (WBC) counts less than 0.5×10^3/mcL (μL) when performed in the low WBC mode. The instrument can be programmed to automatically perform the WBC count and leukocyte differential in this mode if the WBC count falls below this level. In the low WBC mode, the sample is analyzed three times longer to accumulate more cell events and evaluated in the WDF channel, thus improving the WBC count and differential's accuracy.

Within the white cell nucleated (WNR) channel, the leukocyte (WBC) count is determined and basophils and nucleated erythrocytes are clearly differentiated from other leukocytes to determine the number of basophils and nucleated erythrocytes within each blood sample. In the WNR channel, lyse reagent lyses the erythrocytes and removes the nucleated erythrocyte's cytoplasmic membrane. For the leukocytes, lyse reagent perforates the cytoplasmic membrane and alters the leukocyte's external shape and internal structure depending on the characteristics of the specific leukocyte (e.g., segmented neutrophil versus basophil). The fluorescent dye (polymethine) binds to the bare nuclei of the nucleated erythrocytes and enters the leukocytes to bind to nucleic acids and intracytoplasmic organelles. The cells are then analyzed by fluorescent flow cytometry as in the XE-series™ instrument to determine each cell's forward scatter (cell size) and fluorescent intensity (amount of bound fluorescent dye). These data are used to determine the total leukocyte (WBC) count and generate the WNR scattergram (Figure 39-19 ■). Nucleated erythrocytes and basophils can be clearly delineated on this scattergram from other leukocytes and enumerated. Because the delineation between nucleated erythrocytes and leukocytes is clear, the total leukocyte (WBC) count does not require correction for their presence.

The fluorescent platelet (PLT-F) channel is a separate channel that determines the IPF and a platelet count (i.e., PLT-F count). Within this channel, the aspirated blood sample is diluted, and a fluorescent dye (oxazine) is added to the dilution. The oxazine binds to the nucleic acid content of the platelet organelles and binds only diffusely to the reticulocytes. The dilution is then analyzed by fluorescent flow cytometry to determine each cell's forward scatter and fluorescent intensity. Using this information, the PLT-F scattergram is generated (Web Figure 39-6). Platelets are clearly delineated from other cells including reticulocytes based on their size and fluorescent intensity as depicted in the PLT-F scattergram. The IPF is determined from this scattergram because

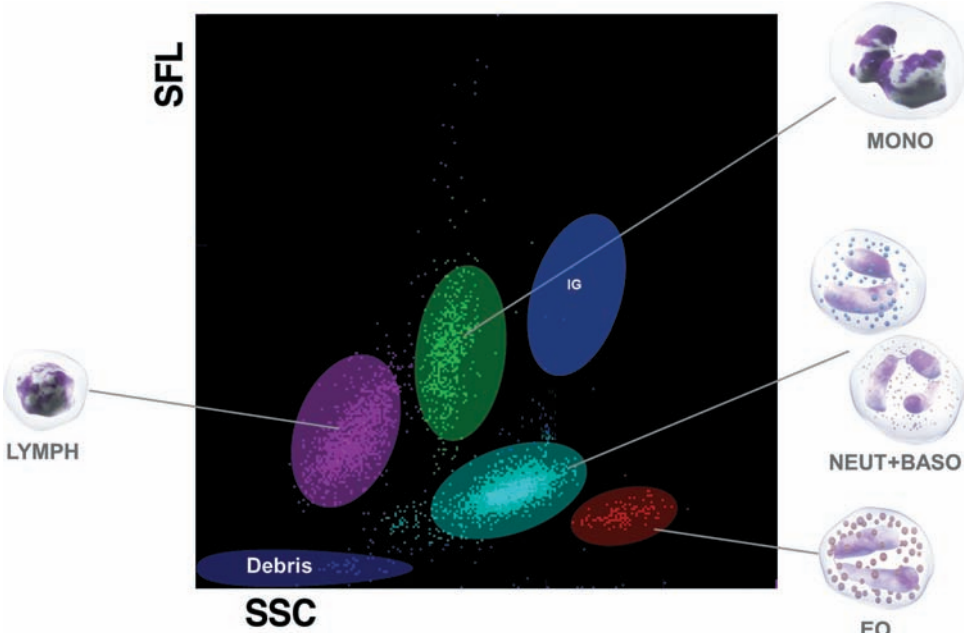


FIGURE 39-18 WDF scattergram, Sysmex XN-Series™. Lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), neutrophils (NEUT) + basophils (BASO), and immature granulocytes (IG) are identified. Basophils are specifically identified in the WNR scattergram.

SSC = side scatter; SFL = fluorescence intensity

Courtesy of Sysmex Corporation, Kobe, Japan.

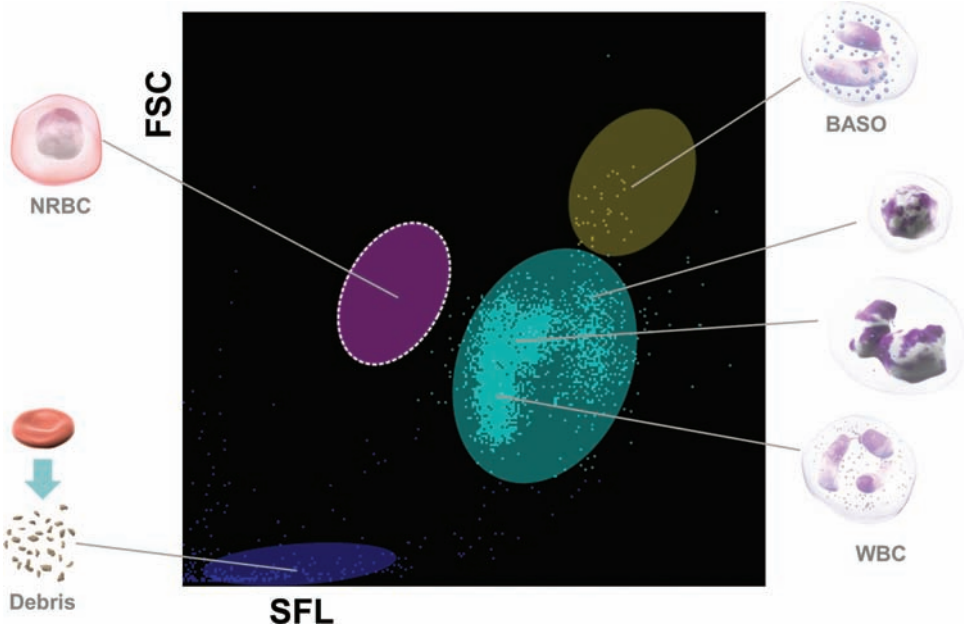


FIGURE 39-19 WNR scattergram, Sysmex XN-Series™. Nucleated erythrocytes and basophils are clearly delineated from other leukocytes (e.g., neutrophils, lymphocytes, and monocytes). Therefore, the total WBC count does not require correction for the presence of nucleated erythrocytes.

Courtesy of Sysmex Corporation, Kobe, Japan.

these cells have high fluorescence intensities. The PLT-F channel can be included as part of the routine measurement for all blood samples, or the user can program it to be a reflex test and performed only when the analyzer onboard rule system detects an abnormal platelet histogram or low platelet count (predetermined by the user). The PLT-F channel provides an accurate platelet count when numbers are low or when potential interferences such as erythrocyte or leukocyte fragments are present.

☑ CHECKPOINT 39-6

Which cellular characteristics are used to determine the IPF on the Sysmex XN-Series™ instrument?

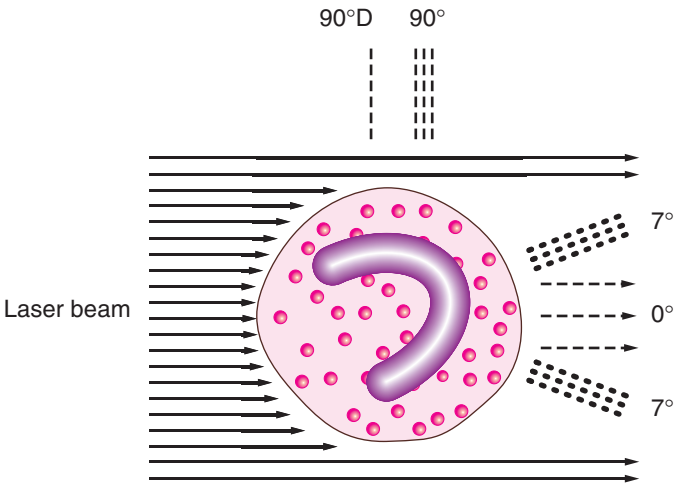
The XN-Series™ have a body fluid module for counting erythrocytes, leukocytes, and determining a two-part differential for all body fluids.[43] The new methods introduced for the leukocyte count and leukocyte differential have also improved the instrument's ability to perform cell counts and cell differentials on body fluids. No pretreatment or off-line preparation is required for body fluid analysis. The erythrocyte count (RBC-BF) is determined by impedance in the RBC/platelet channel, whereas the leukocyte count (WBC-BF), total cell count (TC-BF), and two-part differential (polymorphonuclear cells and mononuclear cells) are determined by fluorescent flow cytometry (side scatter and fluorescent intensity) in the WDF channel. The BF scattergram depicts the polymorphonuclear cells (PMNs) and mononuclear cells (MNs) by their characteristic side scatter and fluorescent intensity patterns (Web Figure 39-7). High-fluorescent intensity body fluid (HF-BF) cells (e.g., macrophages, mesothelial cells) can also be seen on this scattergram. The WBC-BF count represents the number of PMNs plus MNs, whereas the TC-BF count includes WBC-BF count plus the HF-BF cells identified by the BF-WDF scattergram.

Abbott CELL-DYN Sapphire®

Like other automated cell counting instruments, the CELL-DYN Sapphire® uses a combination of technologies to enumerate erythrocytes, leukocytes, platelets, and reticulocytes and determine a five-part leukocyte differential.[44,45] Flow cytometry, fluorescence staining, and impedance are used to make these determinations.

The instrument aspirates a sample of EDTA-anticoagulated blood and sends a portion of this sample to the hemoglobin dilution cup. The hemoglobin reagent dilutes the blood sample, lyses erythrocytes, and converts free hemoglobin to a single chromogen by forming a complex with imidazole. The hemoglobin concentration is determined spectrophotometrically at 540 nm. A reagent blank is used to minimize optical interferences, whereas leukocyte interference is minimal because the reagent destroys leukocytes and cellular fragments. This cyanide-free reaction shows good correlation with the cyanmethemoglobin reference method.

In the WBC dilution cup assembly, the sample is diluted for enumerating total leukocyte (WBC) count, detecting and quantitating nucleated erythrocytes, and determining the five-part leukocyte differential. The preheated WBC reagent dilutes the leukocytes, lyses erythrocytes, strips the cytoplasmic membrane from nucleated erythrocytes and fragile leukocytes, and stains DNA of the exposed nuclei with propidium iodide (PI), a fluorescence dye. This dilution is sent to the optical flow cell. Hydrodynamic focusing directs cells through the flow cell in single file. A solid-state laser interacts with each intact cell or exposed nucleus to create light scatter and fluorescence (PI excites at 488 nm and emits at 630 nm). Using multi-angle polarized scatter separation (MAPSS™) technology, the following light scatter characteristics are determined as indicated: (1) 0° light scatter or forward scatter that reflects cell size, (2) 7° light scatter that reflects cell complexity, (3) 90° light scatter or side scatter that reflects nuclear lobularity, and (4) 90° depolarized light scatter (90°D) that reflects cytoplasmic granularity (Figure 39-20 ■). MAPSS™ technology-generated data are used in different ways to classify leukocyte subpopulations and identify certain morphologic flags.[44] For example, discriminant line analysis isolates and identifies various cell populations. A histogram is created based on one-dimensional data (e.g., size) or two-dimensional data (e.g., size versus DNA content). The data management system identifies the valley between the cell populations and sets a discriminant line. Scatterplots and contour plots of the data are used to further classify subpopulations of leukocytes. The 0° (size) versus 7° (complexity) scatterplot allows differentiation of neutrophils, monocytes, and lymphocytes (Figure 39-21 ■). Eosinophils are differentiated from neutrophils in the 90°D (granularity) versus 90° (lobularity) scatterplot (Web Figure 39-8). Finally, lymphocytes are separated from basophils based on size and complexity. The data management system analyzes these characteristics to determine the total WBC count and the five-part leukocyte differential. Additional information obtained from the evaluation of this dilution includes the enumeration of nucleated erythrocytes and identification of nonviable or fragile leukocytes, which are based on light scatter characteristics and fluorescence intensity (i.e., DNA content). Because the nucleated erythrocytes are clearly distinguished from leukocytes in this dilution, the total WBC count and five-part differential are unaffected by their presence.



■ **FIGURE 39-20** Four light scatter measurements from the CELL-DYN Sapphire®. The 0° scatter reflects cell size; 7° scatter reflects cell complexity; 90° scatter reflects nuclear lobularity; and 90° depolarized scatter reflects cytoplasmic granularity.

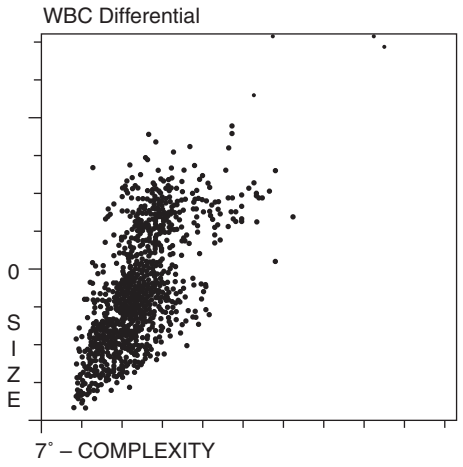


FIGURE 39-21 0° (Size) vs 7° (complexity) scatterplot, CELL-DYN Sapphire®. Neutrophils, monocytes, and lymphocytes are identified. Lymphocytes are located lower left in the scatterplot; the middle cell population is composed of monocytes; and neutrophils are located upper center in the scatterplot.

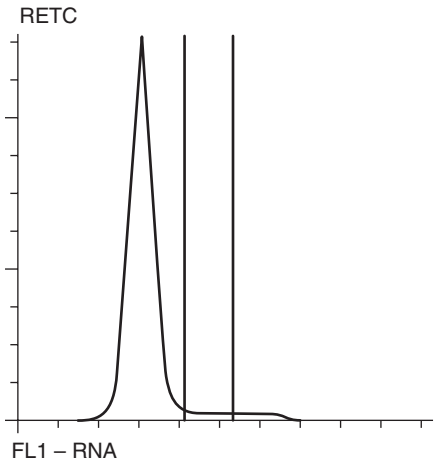


FIGURE 39-22 Reticulocyte histogram, CELL-DYN Sapphire®. Reticulocytes are found between the two gates. The peak to the left represents mature erythrocytes.

x-axis = fluorescence; y-axis = relative number of cells

Two different dilutions are prepared in the RBC/PLT dilution cup assembly. One dilution represents the erythrocyte/platelet dilution. The diluent dilutes the blood sample and spheres the erythrocytes. Using the principles of hydrodynamic focusing and impedance, the erythrocytes and platelets are evaluated as they pass singly through the impedance transducer from which the RBC count, erythrocyte size distribution curve (histogram), platelet size distribution curve, and platelet count are obtained. A second portion of the erythrocyte/platelet dilution is sent to the optical flow cell for enumeration of these cells based on light-scatter characteristics. The RBC count from the optical flow cell and the platelet count from the impedance transducer are used as internal quality control checks against the reported RBC count from the impedance transducer and the reported platelet count from the optical flow cell. The second dilution represents the reticulocyte dilution. The blood sample is diluted with the isotonic diluent, and nucleic acids are stained with a fluorescence dye (i.e., fluorescein isothiocyanate) that excites at 488 nm and emits at 530 nm. This dilution is sent to the optical flow cell where the number of reticulocytes is determined based on fluorescence intensity and light-scatter characteristics (i.e., 7° light scatter). From this information, a reticulocyte histogram is created for the determination of the reticulocyte count and the IRF (Figure 39-22 ■).

The data management system analyzes and compiles all data obtained from the instrument and determines the reported parameters (Web Table 39-1). The results are displayed on a computer screen, printed to a hard copy for the laboratory professional's review (Figure 39-23 ■), or transferred to the LIS. System-initiated messages and data flags alert the laboratory professional to potential abnormalities or errors in the results. This information is used to correlate CBC data with peripheral blood morphology to improve the identification and confirmation of abnormalities.

In 2010, Abbott Diagnostics introduced a new software package for the CELL DYN Sapphire® that extends the number of RBC and reticulocyte parameters generated by this instrument. The data management system calculates these parameters from the data collected during the erythrocyte or reticulocyte enumeration and determines microcytic RBC% (%MIC), macrocytic RBC% (%MAC), hypochromic RBC% (%HPO), hyperchromic RBC% (%HPR), MCVr (reticulocyte mean cell volume), MCHr (reticulocyte mean cell hemoglobin), and CHCr (reticulocyte mean cell hemoglobin concentration). These parameters have been shown to correlate well with similar parameters introduced with the ADVIA hematology instruments (Siemens Healthcare).[46]

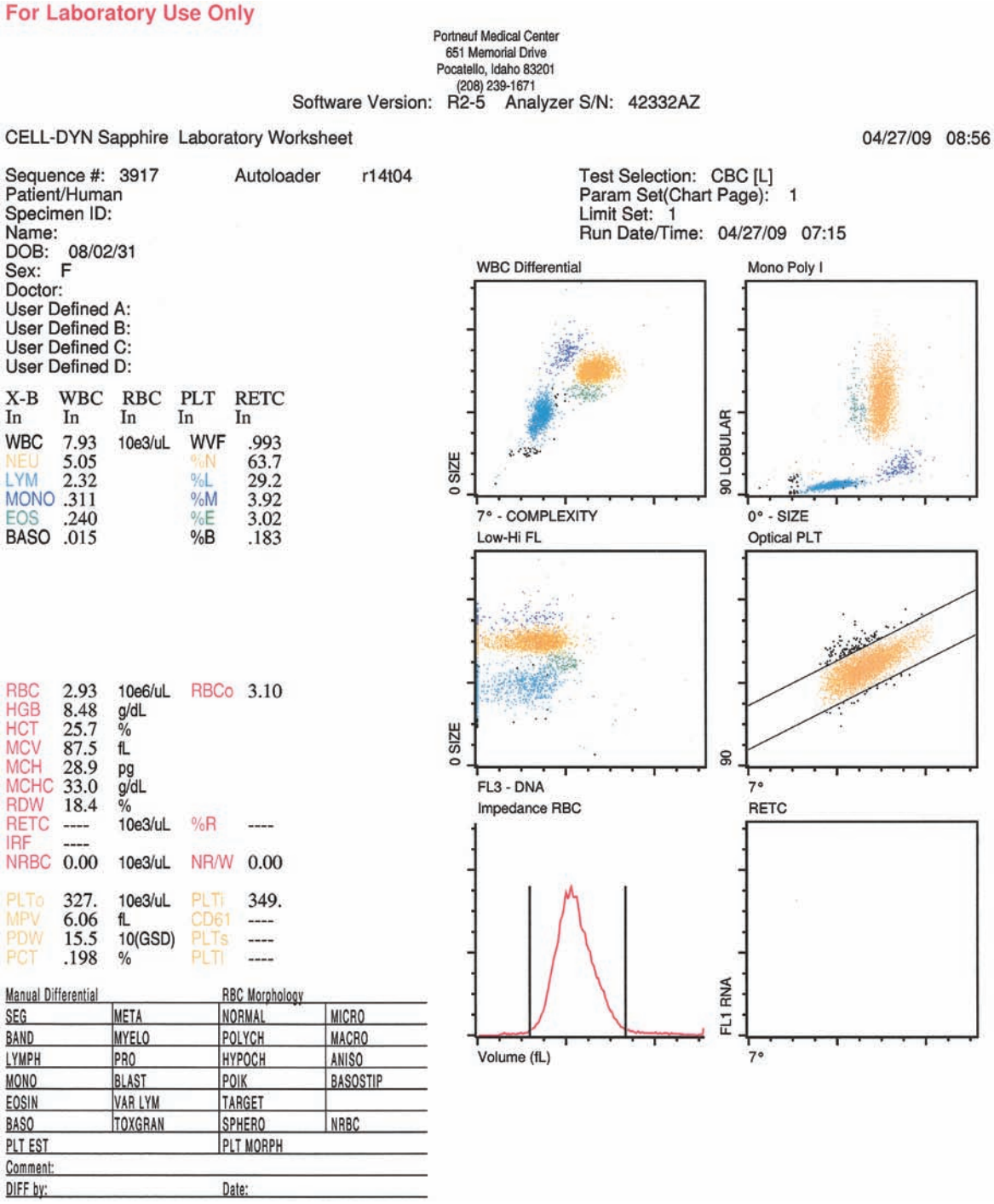
☑ CHECKPOINT 39-7

a. Which CELL-DYN scatterplot allows differentiation of eosinophils from neutrophils?

b. The CELL-DYN Sapphire's WBC dilution contains propidium iodide, so why do the neutrophils not fluoresce?

The CELL-DYN Sapphire® can perform two additional assays, Immuno T-cell (CD3/4/8) Assay, and ImmunoPlt™ (CD61) assay. Both represent fluorescent immunophenotyping methods. The Immuno T-cell (CD3/4/8) Assay uses monoclonal antibodies with different fluorescent labels to identify the T-cell population (i.e., fluorescein isothiocyanate labeled anti-CD3) and its two subpopulations, T helper cells (i.e., phycoerythrin labeled anti-CD4) and T cytotoxic cells (i.e., phycoerythrin labeled anti-CD8). Enumeration of these subpopulations is important in evaluating patients with immunodeficiency syndromes and monitoring certain therapeutic interventions.

The Immuno T-cell (CD3/4/8) Assay consists of two reagent tubes, CD3/CD4 and CD3/CD8. The instrument prepares the reaction mixture in each tube by adding the patient sample and the diluent



For Laboratory Use Only

Portneuf Medical Center
651 Memorial Drive
Pocatello, Idaho 83201
(208) 239-1671

Software Version: R2-5 Analyzer S/N: 42332AZ

CELL-DYN Sapphire Laboratory Worksheet 04/27/09 08:56

Sequence #: 3917 Autoloader r14t04
Patient/Human
Specimen ID:
Name:
DOB: 08/02/31
Sex: F
Doctor:
User Defined A:
User Defined B:
User Defined C:
User Defined D:

Test Selection: CBC [L]
Param Set(Chart Page): 1
Limit Set: 1
Run Date/Time: 04/27/09 07:15

X-B In	WBC In	RBC In	PLT In	RETC In
WBC	7.93	10e3/uL	WVF	.993
NEU	5.05		%N	63.7
LYM	2.32		%L	29.2
MONO	.311		%M	3.92
EOS	.240		%E	3.02
BASO	.015		%B	.183

RBC	2.93	10e6/uL	RBCo	3.10
HGB	8.48	g/dL		
HCT	25.7	%		
MCV	87.5	fL		
MCH	28.9	pg		
MCHC	33.0	g/dL		
RDW	18.4	%		
RETC	----	10e3/uL	%R	----
IRF	----			
NRBC	0.00	10e3/uL	NR/W	0.00

PLTo	327.	10e3/uL	PLTi	349.
MPV	6.06	fL	CD61	----
PDW	15.5	10(GSD)	PLTs	----
PCT	.198	%	PLTI	----

Manual Differential		RBC Morphology	
SEG	META	NORMAL	MICRO
BAND	MYELO	POLYCH	MACRO
LYMPH	PRO	HYPOCH	ANISO
MONO	BLAST	POIK	BASOSTIP
EOSIN	VAR LYM	TARGET	
BASO	TOXGRAN	SPHERO	NRBC
PLT EST		PLT MORPH	
Comment:			
DIFF by:		Date:	

■ **FIGURE 39-23** CELL-DYN Sapphire® report depicting WBC differential.

to the tube's monoclonal antibodies. The instrument rocks the reagent tube gently, incubates the reaction mixture for 2 minutes at room temperature, and then aspirates a portion of the reaction mixture from the tube and places it in the WBC dilution cup. WBC reagent is added to create a final dilution of 1:36, which is sent to the optical flow cell for light scatter and fluorescence intensity measurements. Fluorescence intensity, 0° light scatter (i.e., size), and 7° light scatter (i.e., cell complexity) are used to identify and enumerate the different lymphocyte populations. The scatterplot of 0° light scatter versus 7° light scatter is used to isolate the lymphocyte population for further examination. The scatterplot of CD3 fluorescence intensity (measured at fluorescein isothiocyanate's emission wavelength of 530 nm) versus CD4 fluorescence intensity (measured at phycoerythrin's emission wavelength of 630 nm) determines the percentage of T helper cells; likewise, the scatterplot of CD3 fluorescence intensity versus CD8 fluorescence intensity determines the percentage of T cytotoxic cells (Web Figure 39-9).

The total CD3 fluorescence intensity on a given scatterplot reflects the percentage of T cells present in the patient sample. Comparison of the percentage of CD3 + cells from each scatterplot can be used as an internal control. The computer system uses established algorithms to process the light scatter and fluorescence measurements and generate the reported parameters. These parameters include total T-cell count, percentage of T cells, absolute T helper cell count, percentage of T helper cells, absolute T cytotoxic cell count, percentage of T cytotoxic cells, and the ratio of T helper to T cytotoxic cells. The concentration of these cells can be determined because a CBC is performed on the patient sample prior to preparation of the assay's reagent tubes. This CBC also provides a check of the lymphocyte's viability.

The ImmunoPlt™ (CD61) assay uses a platelet-specific monoclonal antibody, anti-CD61, which is labeled with fluorescein isothiocyanate. This assay is useful when a patient's platelet count is very low (e.g., $<10 \times 10^3$/mcL) or when leukocyte or erythrocyte fragments within the patient sample interfere with platelet enumeration. Because of the specificity of this assay, it provides an accurate platelet enumeration. Like the T-cell assay, the monoclonal antibody is present within a reagent tube. Patient sample and diluent are added to this tube, which are gently mixed. The reagent tube incubates for 1 minute at room temperature. At this point, additional diluent is added to the reagent tube to further dilute the mixture. A portion of this reaction mixture is aspirated into the RBC/platelet dilution cup and diluent is added to create a final dilution of 1:290. A portion of the final dilution is injected into the optical flow cell for measurement. Measurements include 0° light scatter, 7° light scatter, and fluorescence intensity at 530 nm (i.e., fluorescein isothiocyanate's emission wavelength). The instrument's computer system uses established algorithms to process the data generated by these measurements and determines the PLT count. Because of CD61's specificity for platelets, other cells or cell fragments do not affect this platelet count.

Light-Scattering Instruments

Technicon Instruments Corporation was important in the development of hematology instrumentation that used light-scattering technology to enumerate blood cells. Its first instruments were based on **continuous flow analysis** similar to its chemistry instruments. The Hemolog D performed leukocyte differentials based on continuous flow analysis and peroxidase cytochemical staining. The Technicon H-6000 was capable of performing a complete blood cell count and five-part leukocyte differential using continuous flow analysis and an improved cytochemical staining method. The Technicon H *1 was the first of a series of instruments that combined these principles of cell detection and identification with flow cytometry.[47–49] The two instruments described next represent current models whose basic principles of cell enumeration and determination of the leukocyte differential can be traced back to methodologies introduced by the Technicon Instruments Corporation.

Siemens Healthcare ADVIA 120

The ADVIA 120 is capable of performing a CBC, five-part leukocyte differential, and reticulocyte count.[50] It has five measurement channels. The erythrocyte/platelet channel determines the erythrocyte and platelet counts by light-scattering measurements obtained as diluted cells pass singly through a helium-neon laser beam. The diluent used for erythrocyte and platelet counts causes **isovolumetric sphering** of the erythrocytes and platelets. Isovolumetric sphering of erythrocytes eliminates cell volume errors due to variations in erythrocyte shape.[51,52] The erythrocytes are counted and sized by both high-angle (5–15°) and low-angle (2–3°) light-scattering measurements. Individual erythrocyte hemoglobin concentration is determined by the high-angle measurement, and cell volume is determined from the low-angle measurement; thus, the MCV and **cellular hemoglobin concentration mean (CHCM)** are obtained. Together, these measurements are used to generate the erythrocyte cytogram, erythrocyte histogram, and hemoglobin histogram (Figure 39-24 ■). The RDW and the **hemoglobin distribution width (HDW)** are derived from these histograms.

Platelets are evaluated simultaneously with the erythrocytes using both high-angle light scatter and low-angle light scatter; however, these signals are amplified for platelet enumeration. This information is used to create a platelet cytogram, and the actual platelet count is obtained by integrated analysis of the platelet cytogram and erythrocyte cytogram to include large platelets while excluding erythrocytes, erythrocyte fragments, and erythrocyte ghosts.

Within the hemoglobin channel, a portion of the EDTA-anticoagulated blood is mixed with the hemoglobin diluent. The erythrocytes are lysed, and free hemoglobin is converted to cyanmethemoglobin. The concentration of cyanmethemoglobin is determined photometrically at 546 nm.

The leukocyte count and five-part leukocyte differential are obtained utilizing two different methods and two separate channels, the peroxidase and the basophil/lobularity. The peroxidase channel identifies neutrophils, monocytes, eosinophils by the degree of peroxidase positivity (i.e., increased absorption) and the amount of forward light scatter. Lymphocytes and large unstained cells (LUCs) are identified by the amount of forward light scatter and the fact that they remain unstained by this peroxidase cytochemical–staining method. Erythrocytes are removed prior to the peroxidase staining by lytic action. The amount of forward scatter and degree of peroxidase positivity are detected as the cells pass through a tungsten halogen-based flow cell, and this information is used to create the peroxidase cytogram (Web Figure 39-10). Within the basophil/lobularity channel, EDTA-anticoagulated blood is mixed with basophil diluent that lyses erythrocytes and platelets and strips all leukocytes except basophils of their cytoplasm. The helium-neon laser flow cell measures this dilution and determines the degree of high-angle scatter and low-angle scatter for each cell examined. This information is used to create the basophil/lobularity cytogram (Web Figure 39-11). The data management system uses **cluster analysis** to identify individual cell populations within a given cytogram. Each population is identified by its position, area, and density. Thresholds are set, and the number of cells in each population is determined. For the basophil/lobularity cytogram, the normal cell pattern is referred to as the *worm* with the head region representing mononuclear cells and the body region representing polymorphonuclear cells as classified by their high-angle scatter signatures. Basophils have large, low-angle scattered signatures and are located in the region above the worm (Web Figure 39-11). For the peroxidase cytogram, the normal cell pattern depicts the neutrophils in the upper right quadrant, eosinophils in the lower right quadrant, and monocytes in the center triangular region; lymphocytes are located

TEST	NORM	ABN	NORMAL RANGE	UNITS
WBC	8.28		(4.8 –10.8)	x10.e3 /uL
WBCB	8.28		(4.4 –9.9)	
WBCP	8.18		(4.4 –9.9)	
RBC	4.61		(4.15 –5.90)	x10.e6 /uL
HGB	14.0		(12.9 –18.0)	g/dL
HCT	40.3		(40.0 –53.0)	%
MCV	87.4		(83.3 –101.7)	fL
MCH	30.3		(27.0 –33.6)	pg
MCHC	34.6		(30.4 –35.7)	g/dL
CHCM	34.0		(30.4 –35.7)	g/dL
RDW	13.7		(12.6 –15.7)	%
PLT	311		(130 –400)	x10.e3 /uL
MPV	7.7		(7.4 –10.4)	fL
%NEUT	47.4		(25.0 –85.0)	%
%LYMPH	45.0		(10.0 –50.0)	%
%MONO	3.4		(0.0 –15.0)	%
%EOS	1.1		(0.0 –15.0)	%
%BASO	0.3		(0.0 –3.0)	%
%LUC	2.7		(0.0 –6.0)	%
#NEUT	3.93		(1.9 –8.0)	x10.e3 /uL
#LYMPH	3.73		(0.9 –5.2)	x10.e3 /uL
#MONO	0.28		(0.16 –1.0)	x10.e3 /uL
#EOS	0.09		(0.0 –0.8)	x10.e3 /uL
#BASO	0.02		(0.0 –0.2)	x10.e3 /uL
#LUC	0.23		(0 –0.4)	x10.e3 /uL
MPXI	–0.2			
LI	2.17			
%BLASTS	0.2			

Name: _____

SS: _____

Location: _____

MANUAL DIFFERENTIAL

SEGS _____ META _____
BAND _____ MYEL _____
LYMPH _____ PRO _____
MONO _____ BLAST _____
EOS _____ I.M.C. _____
BASO _____ NRBC _____
ATYP _____

RBC MORPHOLOGY

POIK _____ POLY _____
TARGET _____ BURR _____
OVAL _____ TOXIC _____
TEAR _____ DOHLE _____
SCHISTO _____ CREN _____
OTHER _____
COMMENTS:
_____
TECH'S INITIAL _____

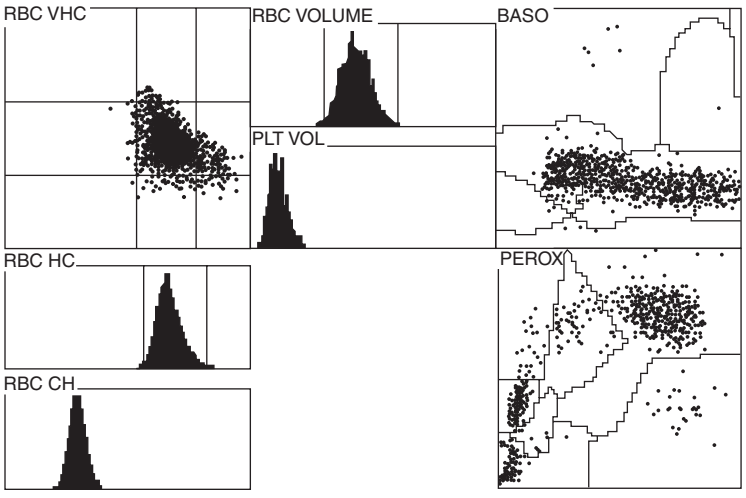


FIGURE 39-24 ADVIA 120 report from a normal individual.

adjacent to the y-axis in the center left quadrant and LUCs are located in the upper left quadrant of the peroxidase cytogram (Web Figure 39-10). The absolute count for each leukocyte population is obtained from the appropriate channel (e.g., absolute neutrophil count from the peroxidase channel and absolute basophil count from the basophil/lobularity channel). The absolute lymphocyte count is obtained by subtracting the absolute basophil count from the absolute lymphocyte count that was obtained from the peroxidase channel. This is necessary because lymphocyte and basophils cannot be differentiated from each other based on analysis of the peroxidase cytogram. The percentage values are calculated from the absolute cell counts. The total WBC count is obtained from both channels. The data management system compares these counts and flags the total WBC count if the results do not compare. An advantage of this method of determining cell counts

is ability to obtain accurate WBC counts and differentials on samples with very low counts (WBC $< 0.1 \times 10^3$/mcL).

The fifth channel is the reticulocyte channel where cellular RNA of the reticulocytes is stained with oxazine 750, a nucleic acid dye. The helium-neon laser flow cell evaluates the erythroid cells for light-scattering and absorbance characteristics. The high-angle light scatter reflects the hemoglobin concentration within the individual erythroid cells, and low-angle light scatter reflects the size of each cell. Reticulocytes are differentiated from mature erythrocytes based on their RNA content, which is determined by the cell's absorbance reading. Reticulocytes have a higher absorbance compared to mature erythrocytes. In addition to the absolute reticulocyte count and relative reticulocyte percentage, reticulocyte analysis includes a measure of the reticulocyte cellular hemoglobin concentration (CHr™), MCVr, and the reticulocyte cellular hemoglobin concentration mean (CHCMr).

The ADVIA 120's data management system evaluates the information from the two flow cells, determines the reported parameters, and displays the information on the computer screen (see Web Table 39-1 for the reported parameters). The results can be printed to hard copy (Figure 39-24) or transferred to the LIS. If abnormalities are detected in cell counts, histograms, or cytograms, the instrument flags the appropriate result(s). The flagging criteria assist the laboratory professional in defining the abnormalities to be reviewed by peripheral blood smear examination.

☑ CHECKPOINT 39-8

What is the similarity in the reticulocyte methods performed on the ADVIA 120 and XE-Series™ instruments?

Siemens Healthcare ADVIA 2120

The ADVIA 2120 represents the latest model in this series of hematology instruments. The basic principles of blood cell enumeration, reticulocyte enumeration, and determination of the five-part leukocyte differential are the same as those discussed in detail for the ADVIA 120. The hemoglobin determination is a cyanide-free method where released hemoglobin forms a stable chromogen with imidazole. This method demonstrates strong correlation with the cyanmethemoglobin method of the ADVIA 120.[53] A disadvantage of the ADVIA 2120 and ADVIA 120 is that neither is currently capable of determining and reporting a nucleated erythrocyte count.

The ADVIA 120 and ADVIA 2120 were the first hematology instruments to introduce extended RBC and reticulocyte parameters (Web Table 39-1). The percentage of hypochromic erythrocytes (%HYPO) and percentage of hyperchromic erythrocytes (%HYPER) are obtained from the red cell erythrogram, a derivation of the erythrocyte cytogram. The percentage of microcytes (%MICRO) and percentage of macrocytes (%MACRO) are derived from the erythrocyte cytogram. Studies indicate that the combined use of %HYPO and CHr™ can be useful in detecting iron deficiency in patients on hemodialysis for chronic renal disease. These patients could experience a superimposed iron deficiency with anemia of chronic disease or have a functional iron deficiency (Chapter 12), which decreases their response to erythropoietin therapy. Early detection of iron deficiency results in appropriate therapeutic intervention to resolve the iron deficiency and, therefore, decrease the poor response rate of hemodialysis patients to erythropoietin therapy.[54–56]

Based on the information provided by the two methods of determining WBC counts and five-part differential, the ADVIA 2120 calculates a new index, the delta neutrophil index (DNI), which is defined as follows: DNI = (neutrophils + eosinophils from the peroxidase channel) minus (polymorphonuclear cells from the basophil/lobularity channel). This index reflects the number of immature granulocytes (i.e., promyelocytes, myelocytes, metamyelocytes) present in the sample. Recent studies reveal that the DNI may be useful as a prognostic indicator for sepsis or an early marker of disease severity in patients with sepsis.[57,58]

The ADVIA 120 and ADVIA 2120 perform CSF analysis using a dedicated CSF mode.[59] The CSF sample is first mixed with the CSF assay reagent and incubated for a minimum of 4 minutes (no longer than 4 hours) to sphere and fix the cells. The instrument aspirates pretreated CSF sample, and determines the cell counts and two-part differential by light scatter and absorbance measurements. The reported results include CSF WBC count, CSF RBC count, and two-part differential. The PMNs and MNs are reported in absolute and relative percentage values.

Summary

This chapter briefly reviewed the ever-increasing uses of technology for the automated hematology laboratory. The blood cell–counting instruments include those using a combination of technologies such as impedance, light scatter, radio frequency, and fluorescent detection to determine the CBC and five-part leukocyte differentials. The two major principles used by hematology instruments are impedance and light scatter. The impedance principle of blood cell counting is based on the increased resistance that occurs when a blood cell with poor conductivity passes through an electrical field. The number of pulses indicates the blood cell count, and the amplitude (i.e., height) of each pulse is proportional to the cell's volume. The optical light–scattering principle of blood cell counting is based on light-scattering measurements obtained as a single blood cell passes through a beam of light (optical or laser). Forward scatter is a measurement of cell size, whereas side scatter is a measure of cell granularity or complexity. Most instruments also measure reticulocytes and nucleated erythrocytes. Reticulocyte evaluation includes not only quantitative data but also data on the immaturity of reticulocytes, which indicates the erythropoietic

response of the bone marrow to therapy for anemia and the hemoglobin content of reticulocytes (CHr™), which indicates the functional availability of iron to the erythron and its incorporation into hemoglobin.

The hematology instruments' data analysis systems examine the data for possible interference or abnormalities and if found, the laboratory professional is alerted by suspect or user-defined flags. The laboratory professional uses the flags to correlate the CBC data with peripheral blood morphology and confirm the abnormalities or correct erroneous results.

To operate these instruments to their fullest potential, it is important that qualified laboratory professionals evaluate the data created by the instrument's analysis of an individual cell's characteristics. Through careful review of that data, new applications of these instruments can arise to aid in the early detection of abnormalities. Automation has increased precision and accuracy within the hematology laboratory and shortened the amount of time needed for analysis, but it has also increased the need for the individual laboratory professional's interpretive skills.

Review Questions

Level I

1. Which automated blood cell–counting instrument uses the optical light scatter method to determine the erythrocyte count? (Objective 3)
   A. Unicel® DxH 800
   B. XE-5000™
   C. CELL-DYN Sapphire®
   D. ADVIA 120
2. Why is hydrodynamic focusing included in the performance of RBC counts by several automated blood cell–counting instruments? (Objective 3)
   A. to ensure that only a single cell enters the detection area at any given time
   B. to direct the beam of light onto the center of the photodetector
   C. to select the appropriate wavelength of light for analysis
   D. to focus the beam of light on the detection area
3. Which parameter does the LH 780 instrument calculate? (Objective 5)
   A. erythrocyte count
   B. MCV
   C. reticulocyte percentage
   D. absolute neutrophil count
4. Which dye is used to stain cellular RNA for reticulocyte counting on the ADVIA 120? (Objective 6)
   A. thiazole orange
   B. oxazine 750
   C. new methylene blue
   D. auramine-O
5. Which automated blood cell–counting instrument has a dedicated channel for nucleated erythrocyte determination? (Objective 3)
   A. CELL-DYN Sapphire®
   B. XE-5000™
   C. LH 780
   D. ADVIA 2120
6. The ADVIA 2120's determination of the %HYPO parameter is: (Objective 5)
   A. calculated using the CHCM and MCV
   B. derived from hemoglobin histogram
   C. calculated using the MCH and erythrocyte count
   D. derived from erythrocyte cytogram
7. Which technology does the XE-5000™ use to determine a leukocyte count on a body fluid? (Objective 3)
   A. impedance
   B. conductivity
   C. fluorescent flow cytometry
   D. optical density
8. Which parameter does the CELL-DYN Sapphire® directly measure? (Objective 5)
   A. hematocrit
   B. platelet count
   C. relative neutrophil percentage
   D. absolute reticulocyte count

9. Which automated blood cell–counting instrument does *not* use the analysis of fluorescence intensity and light scatter to determine the reticulocyte count? (Objective 6)
   A. XE-2100™
   B. ADVIA 120
   C. CELL-DYN Sapphire®
   D. Unicel® DxH 800
10. Which parameter does the Sysmex XE-2100™ derive from a histogram or scattergram? (Objective 5)
   A. relative monocyte percent
   B. erythrocyte count
   C. mean cell volume
   D. hematocrit

Level II

1. What information is needed to create an erythrocyte histogram? (Objective 1)
   A. cell volume and relative cell number
   B. cell size and cell complexity
   C. nuclear size and cellular density
   D. cell forward scatter and cell side scatter
2. The Sysmex XE-2100™ uses all of the following technologies to determine its five-part leukocyte differential *except*: (Objective 1)
   A. fluorescence
   B. radio frequency
   C. optical light scatter
   D. differential cell lysis
3. Using the ADVIA 2120 instrument, which leukocyte cell type is located in the body of the worm of the basophil/lobularity cytogram? (Objective 1)
   A. basophil
   B. monocyte
   C. neutrophil
   D. lymphocyte
4. The Coulter® LH 750 five-part leukocyte differential is determined by the analysis of cellular characteristics as defined by: (Objective 1)
   A. light scatter, cytochemical staining, and radio frequency
   B. light scatter and radio frequency
   C. impedance and cytochemical staining
   D. impedance, conductivity, and light scatter
5. The CHr™ is determined by measuring a cell's: (Objective 2)
   A. absorbance and light scatter characteristics
   B. absorbance and radio frequency characteristics
   C. fluorescence intensity and impedance characteristics
   D. fluorescence intensity and conductivity characteristics
6. The CELL-DYN Sapphire® determines the number of T helper lymphocytes within a blood sample by immunophenotyping and: (Objective 1)
   A. radiofrequency opacity
   B. absorbance spectrophotometry
   C. impedance technology
   D. flow cytometry
7. The IRF reported by the Sysmex XE-2100™ represents the following combined parameters: (Objective 2)
   A. HFR and LFR
   B. LFR and MFR
   C. HFR and MFR
   D. absolute and relative reticulocyte counts
8. The advanced flagging system of the Sysmex XE-5000™ has the potential to: (Objective 1)
   A. increase the number of peripheral blood smear reviews
   B. eliminate the peripheral blood smear reviews
   C. decrease the number of peripheral blood smear reviews
   D. increase the number of errors in blast cell identification
9. Which automated blood cell–counting instrument reports the number of immature granulocytes as part of its leukocyte differential based on analysis of its white cell scattergram? (Objective 1)
   A. CELL-DYN Sapphire®
   B. XN-Series™
   C. Unicel® DxH 800
   D. ADVIA 2120
10. The CHCr determined by the CELL-DYN Sapphire® correlates with which of these parameters on the ADVIA 120: (Objective 2)
   A. CHCMr
   B. MCVr
   C. CHr™
   D. Ret He

Companion Resources

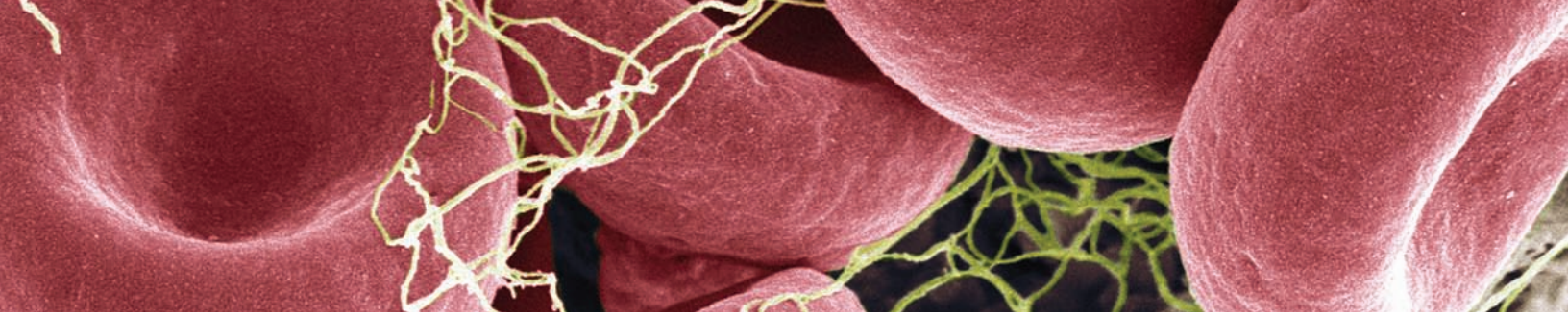
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Mattern CFT, Brackett FS, Olson B. Determination of number and size of particles by electrical gating: blood cells. *J Appl Physiol*. 1957;10:56–70.
2. Jovin TM et al. Automatic sizing and separation of particles by ratios of light scattering intensities. *J Histochem Cytochem*. 1976;24:269–83.
3. Molina FR, Sanchez-Garcia F, Torres A et al. Reticulocyte maturation parameters are reliable early predictors of hematopoietic engraftment after allogeneic stem cell transplant. *Biol Blood Marrow Transplant*. 2007;13:172–82.
4. Brugnara C, Schiller B, Moran J. Reticulocyte hemoglobin equivalent (Ret He) and assessment of iron-deficient states. *Clin Lab Haematol*. 2006;28:303–08.
5. Parodi E, Giraudo MT, Davitto M et al. Reticulocyte parameters: markers of early response to oral treatment in children with severe iron-deficiency anemia. *J Pediatr Hematol Oncol*. 2012;34:e249–52.
6. Miwa N, Akiba T, Kimata N et al. Usefulness of measuring reticulocyte hemoglobin equivalent in the management of haemodialysis patients with iron deficiency. *Int J Lab Haematol*. 2010;32:248–55.
7. Mateos ME, De la Cruz J, Lopez-Laso E et al. Reticulocyte hemoglobin content for the diagnosis of iron deficiency. *J Pediatr Hematol Oncol*. 2008;30:539–42.
8. Paris A, Nhan T, Cornet E et al. Performance evaluation of the body fluid mode on the platform Sysmex XE-5000 series automated hematology analyzer. *Int J Lab Haematol*. 2010;32:539–47.
9. *Coulter System Reference*. Miami, FL: Coulter Corporation;1996.
10. *Coulter STKS Operator's Guide*. Hialeah, FL: Coulter Corporation;1991.
11. Allen JK, Batjer JD. Evaluation of an automated method for leukocyte differential counts based on electronic volume analysis. *Arch Pathol Lab Med*. 1985;109:534–39.
12. Corberand JX. Discovery of unsuspected pathological states using a new hematology analyser. *Med Lab Sci*. 1991;48:80–3.
13. Poulsen KB, Bell CA. Automated hematology: comparing and contrasting three systems. *Clin Lab Sci*. 1991;4:16–7.
14. *Clinical Case Studies: Coulter System Enhanced VCS Technology*. Brea, CA: Beckman Coulter; 2000. Bulletin 9165.
15. *Coulter LH 780 System Reference*. Brea, CA: Beckman-Coulter; 2010.
16. *Red Cell Distribution Parameters—(1) RDW-SD (2) RDW-CV*. Brea, CA: Beckman-Coulter; 2007. Bulletin 9617.
17. Lin CK, Lin JS, Chen SY et al. Comparison of hemoglobin and red blood cell distribution width in the differential diagnosis of microcytic anemia. *Arch Pathol Lab Med*. 1992;116(10):1030–32.
18. *Coulter LH 750 System Reference*. Brea, CA: Beckman-Coulter, 2010.
19. *Unicel DxH 800 Coulter Cellular Analysis System Instructions for Use Manual*. Brea, CA: Beckman-Coulter; 2010.
20. Jean A, Boutet C, Lenormand B et al. The new haematology analyzer DxH 800: an evaluation of the analytical performances and leucocyte flags, comparison with the LH 755. *Int J Lab Haematol*. 2011;33:138–45.
21. Tang H, Jing J, Bo D et al. Biological variations of leukocyte numerical and morphologic parameters determined by Unicel DxH 800 hematology analyzer. *Arch Pathol Lab Med*. 2012;136:1392–396.
22. Hedley BD, Keeney M, Chin-Yee I et al. Initial performance evaluation of the Unicel DxH 800 Coulter cellular analysis system. *Int J Lab Haematol*. 2011;33:45–56.
23. *Sysmex XE-2100 Operator's Manual*. Kobe, Japan: Sysmex; 2006. Revised.
24. Rowan RM, Linssen J. A picture is worth a thousand words. *Sysmex Journal International*. 2005;15(1):27.
25. Briggs C, Harrison P, Grant D et al. New quantitative parameters on a recently introduced automated blood cell counter—XE 2100™. *Clin Lab Haematol*. 2000;22:345–50.
26. Ruzicka K, Veitl M, Thalhammer-Scherrer R, Schwarzinger I. The new hematology analyzer Sysmex XE-2100—performance evaluation of a novel white blood cell differential technology. *Arch Pathol Lab Med*. 2001;125:391–96.
27. Fernandez B, Hamaguchi Y. Automated enumeration of immature granulocytes. *Am J Clin Pathol*. 2007;128:454–63.
28. Nigro KG, O'Riordan M, Molloy EJ et al. Performance of an automated immature granulocyte count as a predictor of neonatal sepsis. *Am J Clin Pathol*. 2005;123:618–24.
29. Mitani N, Yujiri T, Tanaka Y et al. Hematopoietic progenitor cell count, but not immature platelet fraction value, predicts successful harvest of autologous peripheral blood stem cells. *J Clin Apheresis*. 2011;26:105–10.
30. Briggs CJ, Linssen J, Longair I et al. Improved flagging rates on the Sysmex XE-5000 compared with the XE-2100 reduce the number of manual film reviews and increase laboratory productivity. *Am J Clin Pathol*. 2011;136:309–16.
31. Briggs C, Hart D, Kunka S et al. Immature platelet fraction measurement: a future guide to platelet transfusion requirement after haematopoietic stem cell transplantation. *Transfus Med*. 2006;16:101–09.
32. Martinelli G, Merlo P, Fantasia R et al. Reticulated platelet monitoring after autologous peripheral haematopoietic progenitor cell transplantation. *Transfus Apher Sci*. 2009;40:175–81.
33. Yamaoka G, Kubota Y, Nomura T et al. The immature platelet fraction is a useful marker for predicting the timing of platelet recovery in patients with cancer after chemotherapy and hematopoietic stem cell transplantation. *Int J Lab Haematol*. 2010;32:e208–16.
34. Pons I, Monteagudo M, Lucchetti G et al. Correlation between immature platelet fraction and reticulated platelets: usefulness in the etiology diagnosis of thrombocytopenia. *Eur J Haematol*. 2010; 85;158–63.
35. Urrechaga E, Borque L, Escanero J. Potential utility of the new Sysmex XE-5000 red blood cell extended parameters in the study of disorders of iron metabolism. *Clin Chem Lab Med*. 2009;47:1411–416.
36. Urrechaga E, Borque L, Escanero JF. The role of automated measurement of RBC subpopulations in differential diagnosis of microcytic anemia and β–thalassemia screening. *Am J Clin Pathol*. 2011;135:374–79.
37. Urrechaga E, Borque L, Escanero JF. Percentage of hypochromic erythrocytes as a potential marker of iron availability. *Clin Chem Lab Med*. 2012;50:685–87.
38. Evaluation of the new body fluid mode on the Sysmex XE-5000 for counting leukocytes and erythrocytes in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*. 2010;48:665–75.
39. Perne A, Hainfellner JA, Womastek I et al. Performance evaluation of the Sysmex XE-5000 hematology analyzer for white blood cell analysis in cerebrospinal fluid. *Arch Pathol Lab Med*. 2012;136:194–98.
40. Zimmerman M, Ruprecht K, Kainzinger F et al. Automated vs. manual cerebrospinal fluid cell counts: a work and cost analysis comparing Sysmex XE-5000 and the Fuchs-Rosenthal manual counting chamber. *Int J Lab Haematol*. 2011;33:629–37.
41. *Sysmex XN-Series™ Technical Bulletin*. Kobe, Japan: Sysmex; 2012:6–16.
42. Briggs C, Longair I, Kumar P et al. Performance evaluation of the Sysmex haematology XN modular system. *J Clin Pathol*. 2012;65(11):1024–30.
43. Fleming C, Brouwer R, Lindemans J et al. Validation of the body fluid module on the new Sysmex XN-1000 for counting blood cells in cerebrospinal fluid and other body fluids. *Clin Chem Lab Med*. 2012;50(10):1791–798.
44. *CELL-DYN Sapphire Operator's Manual*. Abbott Park, IL: Abbott Laboratories; November 2007.
45. Muller R, Mellors I, Johannessen B et al. European multi-center evaluation of the Abbott Cell-Dyn Sapphire hematology analyzer. *Lab Hematol*. 2006;12(1):15–31.

46. Ermens AAM, Hoffman JJM, Krockenberger M et al. New erythrocyte and reticulocyte parameters on CELL-DYN Sapphire: analytical and preanalytical aspects. *Int J Lab Haematol*. 2012;34:274–82.
47. Watson JS, Davis RA. Evaluation of the Technicon Hematology System. *Lab Med*. 1987;18:316.
48. Bollinger PB, Drewinko B, Brailas CD et al. The Technicon H*1– an automated hematology analyzer for today and tomorrow. *Am J Clin Pathol*. 1987;87:71–8.
49. Nelson L, Charache S, Wingfield S et al. Laboratory evaluation of differential white blood cell count information from the Coulter S-Plus IV and Technicon H-1 in patient populations requiring rapid "turnaround" time. *Am J Clin Pathol*. 1989;91:563–9.
50. *Bayer ADVIA 120 System Operator's Guide*. Tarrytown, NY: Bayer; 1999.
51. Kim YR, Ornstein L. Isovolumetric sphering of erythrocytes for more accurate and precise cell volume measurement by flow cytometry. *Cytometry*. 1983;3:419–27.
52. Mohandas N, Kim YR, Tycko DH et al. Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. *Blood*. 1986;68:506–13.
53. Harris N, Jou JM, Devoto G et al. Performance evaluation of the ADVIA 2120 hematology analyzer: an international multicenter clinical trial. *Lab Hematol*. 2005;11(1):62–70.
54. Buttarello M, Pajola R, Novello E et al. Diagnosis of iron deficiency in patients undergoing hemodialysis. *Am J Clin Pathol*. 2010;133:949–54.
55. Wish JB. Assessing iron status: beyond serum ferritin and transferrin saturation. *Clin J Am Soc Nephrol*. 2006;1(supp 1):S4–8.
56. Tessitore N, Solero GP, Lippi G et al. The role of iron status markers in predicting reponse to intravenous iron in haemodialysis patients on maintenance erythropoietin. *Nephrol Dial Transplant*. 2001;16:1416–423.
57. Park BH, Kang YA, Park MS et al. Delta neutrophil index as an early marker of disease severity in critically ill patients with sepsis. *BMC Infect Dis*. 2011;11:299–308.
58. Seok Y, Choi JR, Kim J et al. Delta neutrophil index: a promising diagnostic and prognostic marker for sepsis. *Shock*. 2012;37:242–46.
59. Mahieu S, Vertessen F, Van Der Planken M. Evaluation of ADVIA 120 CSF assay (Bayer) vs. chamber counting of cerebrospinal fluid specimens. *Clin Lab Haematol*. 2004;26:195–99.



40 Flow Cytometry

FIONA E. CRAIG, MD

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Describe the components of a flow cytometer and the principles of cell analysis.
2. Illustrate by example the clinical applications of flow cytometry.
3. Appraise the use of fluorochrome-labeled antibodies in immunophenotyping by flow cytometry.
4. Give examples of the clinical applications of immunophenotyping by flow cytometry, and interpret single dot plots.
5. Define *clonality* and identify methods for detecting a monoclonal population of cells by immunophenotyping.
6. List the specimens appropriate for immunophenotyping by flow cytometry.
7. Describe how flow cytometry can be used in cell quantitation.
8. Calculate and interpret the absolute CD4 count.
9. Explain how flow cytometry can be applied to DNA analysis.
10. List the cells positive for CD34, and explain the purpose of a CD34 count.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Compare and contrast the immunophenotyping results characteristic of chronic lymphocytic leukemia, hairy cell leukemia, and non-Hodgkin lymphoma.
2. Identify the pitfalls that can be encountered in immunophenotyping mature lymphoid malignancies by flow cytometry, and generate potential solutions.
3. Compare and contrast the immunophenotyping results characteristic of lymphoblastic leukemia (LL) and acute myeloid leukemia.
4. Identify the pitfalls that can be encountered in immunophenotyping acute leukemia and lymphoma by flow cytometry, and generate potential solutions.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

5. Compare and contrast the usefulness of the sucrose hemolysis test, Ham test, and flow cytometry immunophenotyping in diagnosing paroxysmal nocturnal hemoglobinuria (PNH).
6. Select and explain the quality measures that must be enforced in quantitative flow cytometry.
7. Compare and contrast the absolute CD4 count and HIV viral load in the surveillance of HIV infection.
8. Calculate and interpret the S phase fraction and the DNA index, and give the principle of DNA analysis by flow cytometry.
9. Assess the findings of flow cytometry cell analysis using two or more dot plots, and select the most likely cell represented.
10. Evaluate flow cytometry results to identify problems and generate solutions.
11. Summarize the uses of analysis for the CD34 antigen, and choose recommended procedures to analyze this antigen.

Key Terms

Acute undifferentiated leukemia
CD designation
Compensation
DNA Index (DI)
Flow chamber
Fluorochrome
Forward light scatter
Gating
Hematogone
Hydrodynamic focusing
Immunophenotyping
Minimal residual disease
Mixed phenotype acute leukemia
Photodetector
Side light scatter

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I

- Describe the cellular characteristics that differentiate T and B lymphocytes; differentiate the stages of development of granulocytes, lymphocytes, and monocytes. (Chapters 7, 8)
- Summarize the classification of malignant leukocyte disorders. (Chapters 23–28)

Level II

- Summarize the subtypes of malignant disorders and laboratory tests used to help classify them. (Chapter 23)
- Describe the abnormality associated with paroxysmal nocturnal hemoglobinuria (PNH). (Chapter 17)
- Outline the cell cycle. (Chapter 2)

CASE STUDY

We will address this case study throughout the chapter.

Andrew, a 76-year-old male, had a complete blood count (CBC) performed during hospitalization for pneumonia. He was found to have a WBC of 76 $\times$ 10^9/L with 80% lymphocytes.

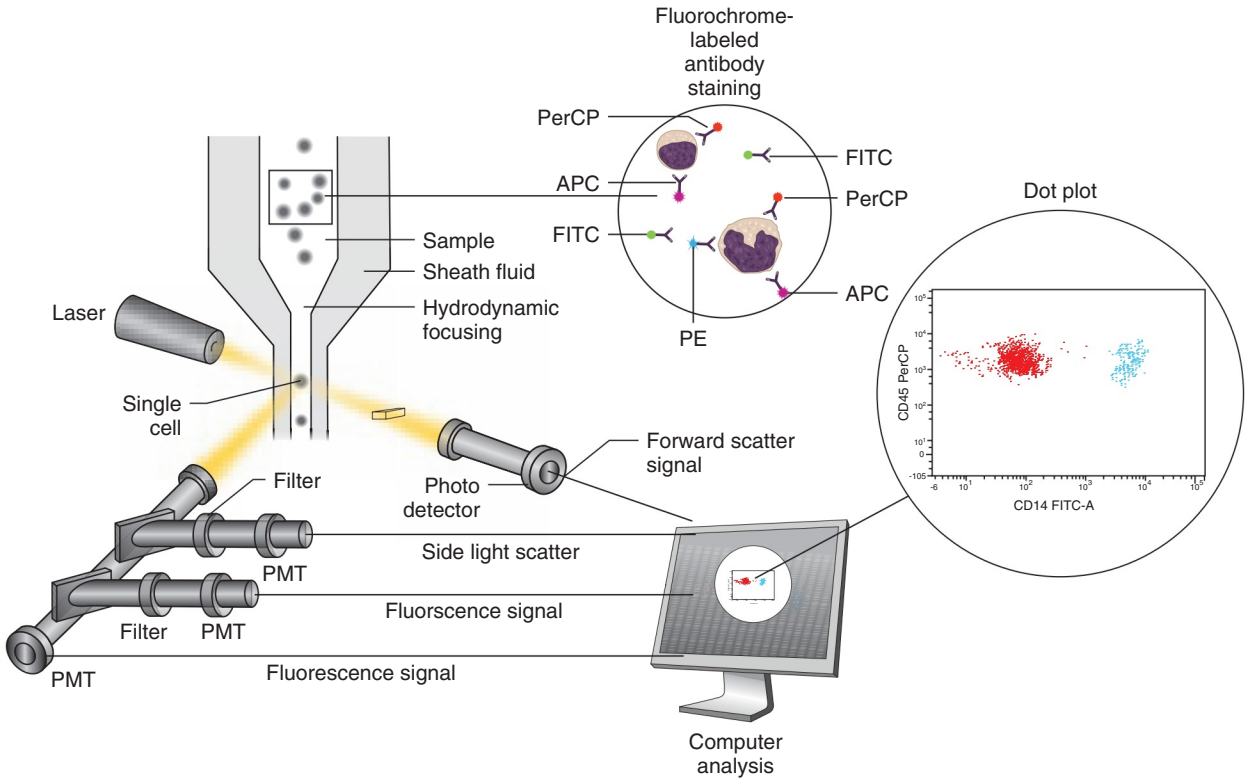
Consider the conditions that are associated with these results and the follow-up testing that might be necessary to confirm a diagnosis.

OVERVIEW

The purpose of flow cytometry is to detect and measure multiple properties so that cells or particles can be identified and quantitated. This chapter introduces the principles of flow cytometry and discusses its clinical applications. First, the method of detecting and quantitating particles/cells by flow cytometry is described. This description includes specimen requirements and processing and the concept of gating to isolate cells of interest. The remainder of the chapter addresses the uses of flow cytometry in the clinical laboratory. Flow cytometry is currently used to analyze individual cells for the presence of antigens (immunophenotyping), count cells with a particular immunophenotype, and quantitate deoxyribonucleic acid (DNA). Immunophenotyping is one of the tools used for diagnosing mature lymphoid malignancies, lymphoblastic leukemia/lymphoma, acute myeloid leukemia (AML), and paroxysmal nocturnal hemoglobinuria (PNH), and monitoring HIV infection. The material in this chapter should be studied with Chapters 26, 27, and 28 (neoplastic disorders) to obtain a full understanding of where flow cytometry fits into the diagnostic workup of these disorders. This chapter also introduces the application of flow cytometry in DNA analysis.

INTRODUCTION

A *flow cytometer* is an instrument capable of detecting molecules on the surface or inside individual particles such as cells. Isolating single particles/cells and labeling the molecule of interest with a fluorescent marker detects molecules (Figure 40-1 ■). Particles/cells that possess the molecule of interest are recognized by the emission of fluorescent light following excitation. Information is acquired from many thousands of particles/cells and stored on a computer



■ **FIGURE 40-1** Flow cytometer. The cells in the specimen are stained with fluorochrome-labeled antibodies and separated into single cells that pass in front of a laser light source. Scattered and emitted light are detected with photodectors and photomultiplier tubes (PMTs), undergo computer analysis, and are displayed as dot plots.

APC = allophycocyanin; FITC = fluorescein isothiocyanate; PE = phycoerythrin; PerCP = peridinin chlorophyll

for further analysis (Figure 40-1). The current applications of flow cytometry in the clinical laboratory include immunophenotyping leukocytes and erythrocytes (identifying antigens using detection antibodies), counting CD4+ and CD34+ positive cells, and analyzing DNA (Table 40-1 ★).[1]

★ **TABLE 40-1** Applications of Flow Cytometry

Application	Examples
Immunophenotyping	Diagnosis and classification of mature lymphoid malignancies
	Prognostic markers in chronic lymphocytic leukemia (CD38 and ZAP-70)
	Diagnosis and classification of acute leukemia
	Detection of minimal residual leukemia following therapy
	Diagnosis of PNH
	Enumeration of T-cell subsets (e.g., CD4 counts in HIV)
	Enumeration of CD34+ progenitor cells for transplantation
RNA analysis	Reticulocyte counting (Chapter 11)
DNA analysis	S phase fraction
	Ploidy

PRINCIPLES OF FLOW CYTOMETRY

Isolation of Single Particles

Flow cytometry is performed on particles in suspension (e.g., cells or nuclei). Leukocytes from peripheral blood and bone marrow specimens are often analyzed after the removal of red blood cells (RBCs) by lysis. The cell suspension is aspirated and injected into a **flow chamber** (Figure 40-1), the specimen-handling area of a flow cytometer where cells are forced into single file and directed into the path of a laser beam.

The flow chamber contains two columns of fluid. The particles/cells are contained in an inner column of sample fluid that is surrounded by a column of sheath fluid (Figure 40-1). The sheath and sample fluids are maintained at different pressures and move through the flow chamber at different speeds. This gradient between the sample and sheath fluid keeps the fluids separate (laminar flow) and is used to control the diameter of the column of sample fluid. The central column of sample fluid is narrowed to isolate single particles/cells that pass through a laser beam (**hydrodynamic focusing**) like a string of beads. Laser light is focused on these single particles/cells and as it is scattered off them, it is measured using **photodetectors** called *photodiodes*. If particles/cells have fluorescent molecules attached, the laser light excites the molecules, which then emit light of a specific wavelength, detected using another type of photodetector called a *photomultiplier tube (PMT)*.

Light Scattering

When the laser beam interacts with a single particle/cell, light is scattered, but its wavelength is not altered. The amount of light scattered in different directions can be used to identify the particle/cell because it is related to the particle's physical properties (size, granularity, and nuclear complexity). Light scattered at a 90° angle (**side light scatter**) is related to the internal complexity and granularity of the particle/cell. Neutrophils produce much side scatter because of their numerous cytoplasmic granules (Figure 40-2 ■). Light that proceeds in a forward direction (**forward light scatter**) is related to the particle's size. Large cells produce more forward scatter than small cells do. Therefore, light scattering can be used to distinguish particles, and several hematology analyzers currently use it to perform differential counting of leukocytes (Chapter 10).

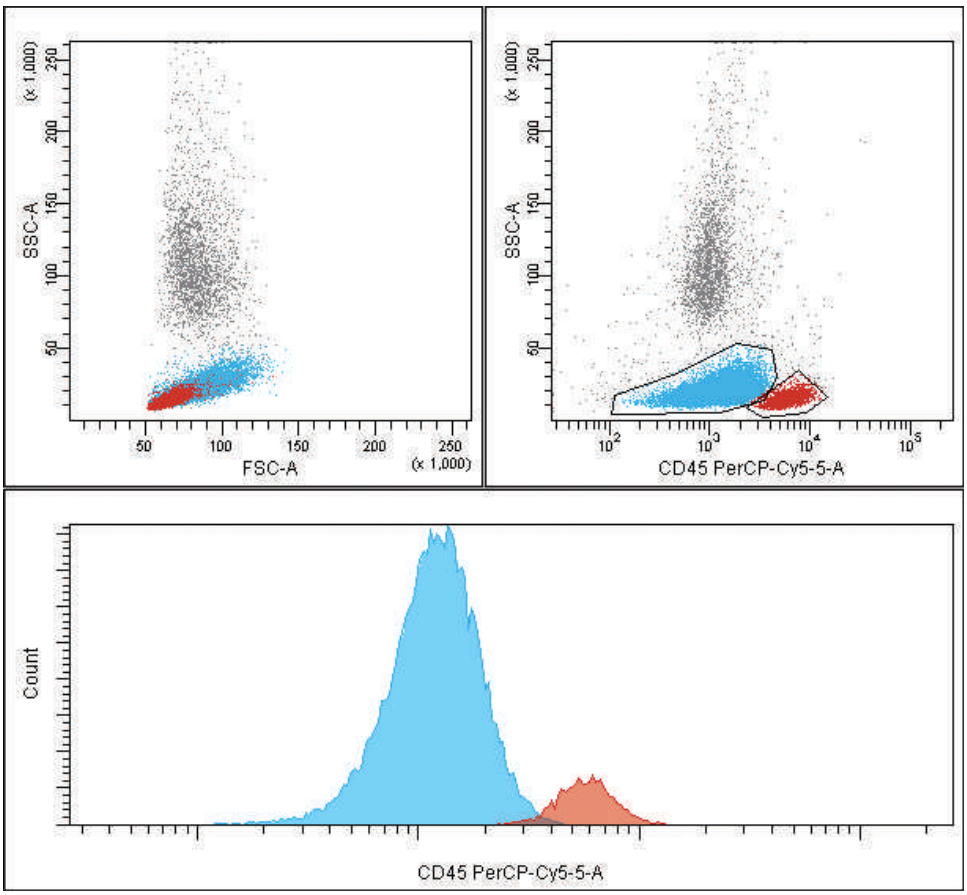
☑ CHECKPOINT 40-1

Would a lymphocyte or monocyte have more forward light scatter?

Detection of Fluorochromes

In addition to light scattering, the flow cytometer can be used to detect bound fluorescent markers (**fluorochromes**), which are molecules that are excited by light of one wavelength and emit light of a different wavelength (fluorescent light). Fluorochromes can be attached to the cells using detection antibodies or bind stoichiometrically to DNA (Table 40-2 ★). Flow cytometers use light of a single wavelength generated by a laser to excite fluorochromes bound to the particle of interest. Light emitted from the fluorochrome is separated from the incident laser light using a combination of filters and mirrors. A PMT then detects and quantifies the emitted light (Figure 40-1).

Clinical flow cytometers usually contain an argon laser that generates light at 488 nm. This single wavelength is often used to excite three different fluorochromes, each emitting light at different wavelengths. Using three different fluorochromes allows detection of three different antigens on the cell. Excitation of additional fluorochromes to detect further antigens usually requires additional laser light sources (e.g., helium neon, emission 633 nm). Currently, clinical immunophenotyping studies frequently utilize two or three laser light



■ **FIGURE 40-2** Flow cytometry histograms. Data acquired using flow cytometry can be displayed in a variety of plots. The plot of forward light scatter (FSC-A) versus side light scatter (SSC-A) (top left) distinguishes granulocytes (black) with high side scatter from other cells but cannot distinguish blasts (blue) from lymphocytes (red). The plot of CD45 versus SSC-A (top right) distinguishes blasts with weak intensity CD45 expression (blue) from lymphocytes with bright intensity CD45 expression (red). Data can also be displayed as single-color histograms (lower plot) but are less useful for distinguishing cells types.

Histograms generated using FACS DIVA software BD Biosciences, San Jose, CA.

★ **TABLE 40-2** Example Fluorochromes Used in Flow Cytometry

Fluorochrome	Excitation Wavelength (nm)	Detection Wavelength (nm)
BD Horizon™ V450	405	450
BD Horizon™ V500	405	500
FITC	488	525
PE	488	575
PE-Cy7	488/561	774
PerCP-Cy5.5	488/675	690
Propidium iodide	488	620
APC	650	660

APC = allophycocyanin; FITC = fluorescein isothiocyanate; PE = R-phycoerythrin

PE-Cy7 is a conjugate of two fluorochromes: PE is excited at 488 nm, and the emitted signal (561 nm) excites the Cy7 component of the molecule. PerCP-Cy5.5 is a conjugate of two fluorochromes: PerCP is excited at 488 nm and the emitted signal (675 nm) excites the Cy5.5 component of the molecule.

V450 (BD Biosciences, San Jose, CA), and V500 (BD Biosciences, San Jose, CA).

sources to identify four to eight antigens on each cell analyzed (4–8 color analysis) (Table 40-2). Some flow cytometers used in a research setting are capable of detecting 18 different fluorochromes.

A fluorochrome unfortunately does not emit light at a single wavelength. Mirrors, filters, and photodiodes are used to detect the peak wavelength of light emitted from each fluorochrome. However, some overlap between the light emitted from different fluorochromes usually exists. For example, although the peak emissions for the two fluorochromes fluorescein isothiocyanate (FITC) and phycoerythrin (PE) are different, PE emits some light at the wavelength used to detect FITC. This overlap is compensated for either by adjusting the settings on the flow cytometer or by performing a mathematical correction either before or after the data are collected. This process is called **compensation**.

Although FDA-approved commercially available kits are available for some specific flow cytometric tests (e.g., CD4 or CD34 enumeration), clinical laboratories are responsible for developing combinations of fluorochrome-labeled antibodies that meet other specific clinical needs (e.g., detection of mature B-cell lymphoid neoplasms). Assay development includes identifying the properties of interest, selecting fluorochrome labeled reagents, titering all reagents, and testing the assay's performance such as precision, sensitivity, and specificity, before implementation.

Data Analysis

Flow cytometry generates a large amount of data, often with at least six parameters measured for each of tens of thousands of particles/cells. Although semiautomated computer analysis programs are available for some applications (e.g., CD4 and CD34 enumeration), clinical laboratory professionals perform most clinical analysis manually. Analysis typically involves identification of the particles/cells of interest, including distinction from debris and perhaps dead cells, and characterization by evaluating all parameters measured. Analysis is performed by plotting the data on graphs that display the presence or absence of the parameter measured and the intensity of the fluorescent signal detected (Figure 40-2). Histograms display information from one parameter versus the number of particles/cells detected with different intensity of emitted light. Two-dimensional (2D) dot plots display information from two different parameters with each particle/cell represented as a single point on the graph. Many 2D dot plots are necessary to view all the possible combinations of parameters evaluated with a single 4–10 color tube.

☑ CHECKPOINT 40-2

A laboratory wants to identify cells that have two different antigens. How many laser light sources are needed?

IMMUNOPHENOTYPING BY FLOW CYTOMETRY

Immunophenotyping is the identification of antigens using detection antibodies. Antibodies are utilized because they bind specifically to antigens and can be labeled with fluorochromes to provide a sensitive and specific detection method. Most flow cytometric immunophenotyping studies involve detecting cell surface antigens. Intracytoplasmic and intranuclear antigens can be detected following permeabilization of cell membranes with detergent and/or alcohol. Detection antibodies can be either polyclonal or monoclonal. Polyclonal antibodies are made by injecting antigen into animals (e.g., rabbit antihuman antibodies). The animal produces many antibodies that are directed against different portions (epitopes) of the antigen. Therefore, the antigen can be recognized even if some parts of it are abnormal. However, polyclonal antibodies are often difficult to standardize and prone to nonspecific binding.

Monoclonal antibodies are directed against a single epitope of the antigen. They are produced in myeloma/tumor cell lines and therefore have high purity and reproducibility. Often several different monoclonal or polyclonal antibodies are available from different suppliers to detect a single antigen and can be given a unique company-specific designation. An international workshop has been developed to systematically review antibodies and group those recognizing the same antigen into a cluster of differentiation (**CD designation**). For example, the commercially available antibodies Leu-4 and OKT3 recognize the same antigen and both are designated CD3. Many fluorochrome-labeled detection antibodies are available commercially (Table 40-3 ★). Combinations of these antibodies are currently used in the clinical laboratory to help identify cells and diseases[2,3] (Appendix B Table).

★ TABLE 40-3 Antibodies Used for Immunophenotyping by Flow Cytometry

CD Designation	Normal Distribution	Use
CD1a	Immature T cell	Lymphoblastic leukemia/lymphoma
CD2	T cell, NK cell	Lineage of lymphoma or leukemia
CD3	T cell	Lineage of lymphoma or leukemia
CD4	T cell	Lineage of lymphoma or leukemia
CD5	T cell	Lineage of lymphoma/leukemia or aberrant expression on B cell SLL/CLL and mantle cell lymphoma
CD7	T cell, NK cell	Lineage of lymphoma or leukemia
CD8	T cell	Lineage of lymphoma or leukemia
CD11c	Monocytes, lymphoid cells	Hairy cell leukemia: bright+; SLL/CLL: dim+
CD11b	Neutrophils	Myelodysplastic syndrome
CD13	Monocytes	Lineage of leukemia
	Myeloid cells	Myelodysplastic syndrome
CD14	Monocytes	Lineage of leukemia
CD15	Monocytes	Lineage of leukemia
	Myeloid cells	
CD16	NK, NK-like T cells	Large granular lymphocyte leukemia
	Granulocytes	Myelodysplastic syndrome
CD19	B cell	Lineage of lymphoma or leukemia
CD20	B cell	Lineage of lymphoma or leukemia
CD22	B cell	Lineage of lymphoma or leukemia, SLL/CLL: dim+; PLL: bright+
CD23	B cell	SLL/CLL+, mantle cell−
CD25	Many cell types	Hairy cell leukemia bright+
CD33	Monocytes	Lineage of leukemia
	Myeloid cells	
CD34	Stem cells, progenitor cells	Stem cells for transplantation, acute leukemia
CD38	Plasma cells, some lymphocytes, monocytes, myeloid cells	Plasma cell neoplasms
		Prognostic marker in CLL
CD42	Megakaryocytes	Acute megakaryocytic leukemia
CD45	All leukocytes	Lineage of malignancy
		Gating
CD55	GPI-anchored protein	Paroxysmal nocturnal hemoglobinuria
CD56	NK, NK-like T cells	Large granular lymphocyte leukemia
CD57	NK, NK-like T cells	Large granular lymphocyte leukemia
CD59	GPI-anchored protein	Paroxysmal nocturnal hemoglobinuria
CD61	Megakaryocytes	Acute megakaryocytic leukemia
CD79a	B cells (blasts to plasma cells)	Lineage of lymphoma or leukemia
CD103	Subset of intramucosal T cells	Hairy cell leukemia
		Enteropathy associated T-cell lymphoma
Glycophorin A	Erythroid	True erythroleukemia
Myeloperoxidase	Myeloid cells	Lineage of leukemia
Kappa	B cell	Maturity, clonality, SLL/CLL dim
Lambda	B cell	Maturity, clonality, SLL/CLL dim

CLL = chronic lymphocytic leukemia; SLL = small lymphocytic lymphoma; PLL = prolymphocytic leukemia; dim = weak intensity of emitted fluorescence; bright = strong intensity of emitted fluorescence; NK = natural killer; GPI = glycosylphosphatidylinositol

☑ CHECKPOINT 40-3

A cell population is positive with both Leu1 and T1 monoclonal antibodies. As a result, the cell is classified as CD5 positive. Explain.

Specimen Requirements and Preparation for Immunophenotyping

Immunophenotyping by flow cytometry requires a suspension of individual live cells (Table 40-4 ★). Anticoagulated blood or bone marrow aspirate, body fluid specimens, and fine needle aspiration samples are ideal for immunophenotyping by flow cytometry because they already contain cells in suspension. Leukocytes can be isolated from these samples either by erythrocyte lysis or density gradient centrifugation. Lysis methods are strongly recommended because leukocytes are retained in their original proportions without the risk of losing cell subtypes. Hematopoietic and lymphoid cells can also be isolated from fresh tissue biopsy specimens by manual disaggregation.

Once a suspension of leukocytes without intact erythrocytes has been prepared, the sample is stained using fluorochrome-labeled antibodies. (In some studies, the leukocytes are stained before the erythrocytes are lysed.) The labeled sample is aspirated into the flow cytometer, and the amount of scattered light and the intensity of each fluorescent signal is recorded for every cell analyzed. The acquired data are then displayed graphically on dot plots (Figure 40-2). Populations of cells that have similar staining properties and can be highlighted with a different color are identified (Figure 40-2). Alternatively, plots are divided into quadrants, indicating cells labeled with one fluorochrome, the other fluorochrome, or both fluorochromes.

The intensity of light emitted is determined by comparison with the known range of intensities for that antibody/fluorochrome combination and divided roughly into three portions: dim or weak intensity, intermediate intensity, and bright or strong intensity (Figure 40-3 ■). The intensity of emitted light is related to the density of antigens.

Isolation of Cells of Interest by Gating

Immunophenotyping requires isolation of the cells of interest (e.g., lymphocytes from monocytes and granulocytes). Cells can be separated during data analysis by placing an electronic gate around those with the same light-scattering or fluorescence properties (**gating**). For example, lymphocytes can usually be separated from neutrophils by their location on the forward versus side light scatter dot plot (Figure 40-2). Fluorescence dot plots can then be set up to display only information obtained from cells falling within the chosen gate. If the gate is too wide, many cell types are included, and identifying the phenotype of the cells of interest becomes difficult. If the gate is too narrow, some cells of interest can be excluded.

The forward versus side light scatter dot plot unfortunately is not capable of separating all the cells of interest. For example, blasts and lymphocytes often appear in the same region on the forward versus side light scatter dot plot. Therefore, a different gating strategy could be required for analyzing acute leukemia. Blasts often have dim CD45 expression, whereas lymphocytes have bright CD45 staining. Therefore, blasts and lymphocytes can be distinguished on a dot plot displaying CD45 intensity versus side angle light scatter (Figure 40-2). Fluorescence staining can be used for gating only if the chosen antibody (CD45) is present in each analysis tube.

★ **TABLE 40-4** Specimen Requirements for Immunophenotyping by Flow Cytometry

Specimen Type	Requirements	Storage
Peripheral blood	5 mL in EDTA or heparin	<24 hrs, RT
Bone marrow	1 mL in EDTA or heparin	<24 hrs, RT
Fluids	as much as possible	<24 hrs, 4°C
Tissue	Preferably > $\frac{1}{2}$ cm^3	<24 hrs, 4°

RT = room temperature; EDTA = ethylenediaminetetraacetic acid

CASE STUDY (continued from page 854)

Flow cytometry immunophenotyping was requested on Andrew.

1. What is the optimal specimen?
2. Which cells are of interest and should be included in the gate?
3. What are the typical forward and side scatter properties of the cells of interest?

☑ CHECKPOINT 40-4

Explain why lymphocytes and neutrophils can be separated on a forward versus side light scatter dot plot.

Diagnosis and Classification of Mature Lymphoid Neoplasms

Flow cytometric immunophenotyping can be used to identify cell lineage (B, T, or natural killer [NK] cell) and the presence of an abnormal population of lymphocytes. This information can assist in detecting malignant cells and identifying a subtype of malignancy. Neoplasms are made up of a population of identical cells (clone). A clone of B lymphocytes can be recognized by uniform expression of one immunoglobulin light chain (κ or λ light chain class restriction). In contrast, a reactive population of B lymphocytes contains a mixture of cells, each with expression of either κ or λ immunoglobulin light chain.

Clonality can also be detected by identifying a population of cells that display an abnormal phenotype (aberrant expression of a

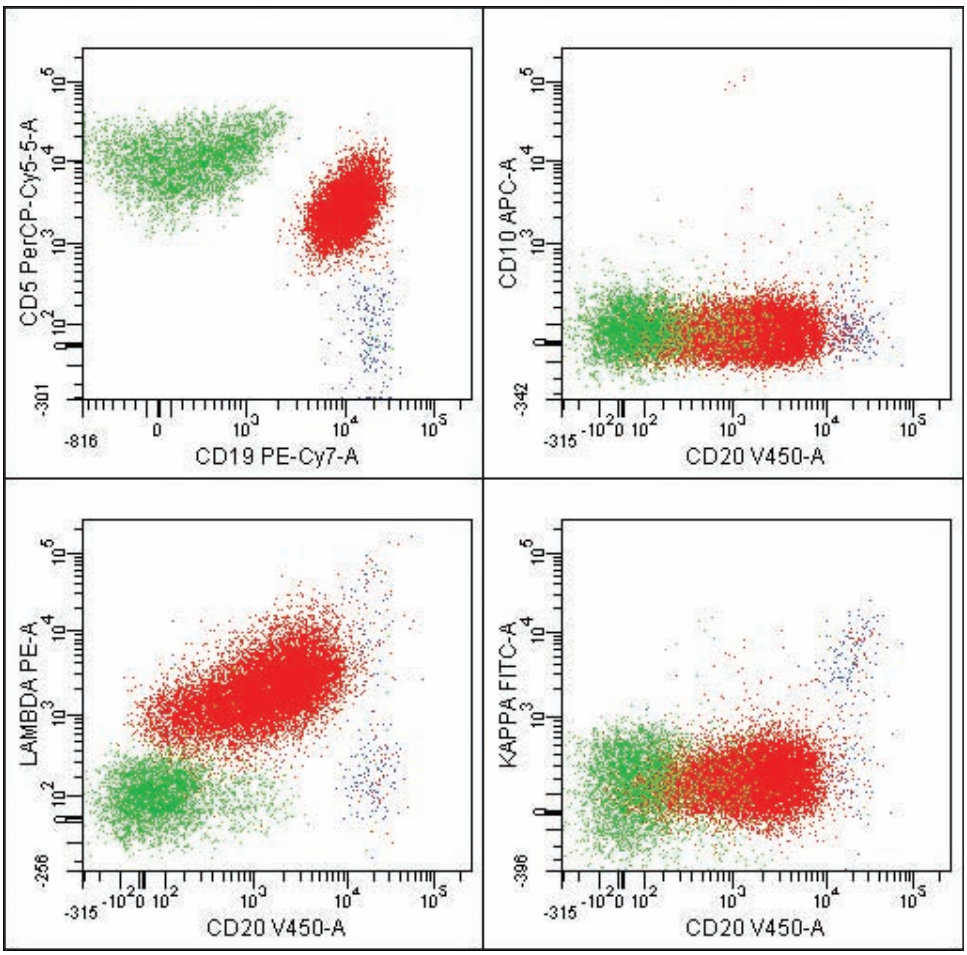


FIGURE 40-3 Flow cytometry histograms from analysis of peripheral blood from Andrew. Cells with low side light scatter were gated, and staining with a number of fluorochrome-labeled antibodies were displayed. The population of interest (red) demonstrates staining for CD19, CD20 (weak intensity), CD5, and λ light chain, but lacks staining for CD10. The plots also display T cells (green) and a few polytypic B cells (blue).

Histograms generated using FACS DIVA software BD Biosciences, San Jose, CA.

lymphoid antigen). For example, mature T lymphocytes normally express CD3, CD2, CD5, and CD7. A clone of malignant T lymphocytes could lack detectable CD5 and/or CD7. Another example of an abnormal phenotype is acquisition of an antigen that is not normally present. For example, the T-cell marker CD5 is aberrantly expressed on a subset of B-cell malignancies (small lymphocytic lymphoma/chronic lymphocytic leukemia and mantle cell lymphoma) (Figure 40-3). Therefore, the presence of CD5+ B lymphocytes can assist in identifying a lymphoid malignancy and diagnosing a specific subtype. Panels of antibodies are usually selected to separate the common subtypes of lymphoproliferative disorders (Table 40-5 ★). In addition, flow cytometric immunophenotyping can be used to demonstrate the presence of antigens for directed therapy (for example, CD20 for anti-CD20 monoclonal antibody therapy), identify prognostic markers such as ZAP-70 expression in chronic lymphocytic leukemia (CLL), and identify a small population of abnormal cells following treatment (**minimal residual disease**).[4]

CASE STUDY *(continued from page 859)*

Flow cytometry revealed the results displayed in Figure 40-3.

4. What is the phenotype?
5. Which features indicate clonality?
6. What is the diagnosis?

Although flow cytometric immunophenotyping usually provides useful information, potential pitfalls can lead to an incorrect diagnosis. A malignant B-cell lymphoproliferative disorder is easily overlooked if it lacks surface immunoglobulin. Some lymphoid malignancies frequently lack diagnostic surface antigens, including plasma cell neoplasms, HIV-associated lymphoma, and mediastinal lymphoma.

★ **TABLE 40-5** Characteristic Immunophenotype of Lymphoproliferative Disorders

Diagnosis	CD19	CD5	CD23	CD11c	CD22	CD25	sIg
Chronic LL	+	+	+	+/−	+w	+/−	+w/−
Prolymphocytic leukemia	+	−/+	−	−	+	+/−	+
Hairy cell leukemia	+	−	−/+	+br	+br	+	+
Small lymphocytic lymphoma	+	+	+	+/−	+w	+/−	+w/−
Mantle cell lymphoma	+	+	−	−	+	−	+
Follicular lymphoma	+	−	+/−	−	+	−	+

br = bright or strong fluorescence intensity; w = weak or dim fluorescence intensity; + = antigen present; − = antigen absent; +/− = variable expression (often present); −/+ = variable expression (often absent).

T-cell lymphoma can be difficult to detect because many cases do not demonstrate an abnormal phenotype. Hodgkin lymphoma is difficult to detect by flow cytometry for several reasons: the neoplastic cells are rare and lack many cell surface lymphoid antigens, and a single cell suspension is often difficult to produce because of the presence of fibrosis. Another potential pitfall in flow immunophenotyping is the presence of an abnormal phenotype that is not specific for a single subtype of lymphoid malignancy. For example, CD10 expression can be seen in follicular lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma. Therefore, it is important to interpret immunophenotyping data in conjunction with morphology.

Diagnosis and Classification of Acute Leukemia

Using the World Health Organization (WHO) classification, a diagnosis of acute leukemia requires manual differential counting to identify more than 20% blasts in the peripheral blood or bone marrow (Chapter 23). Once a diagnosis of acute leukemia has been established, flow cytometry can be used to assist in identifying its subtype. Currently, most treatment protocols require the distinction of lymphoblastic leukemia (LL; also known as *acute lymphocytic leukemia [ALL]*) and *acute myeloid leukemia (AML)*. The recognition of Auer rods or the presence of staining with cytochemical stains allows the identification of AML (Chapters 23 and 26). Until the advent of immunophenotyping, all cases of acute leukemia lacking these features were assumed to be ALL. However, immunophenotyping studies have revealed that this assumption is erroneous. Some AML cases lack cytochemical staining and are recognized only by immunophenotyping (AML not otherwise specified [NOS] with minimal differentiation). In addition to the accurate separation of LL from AML, flow cytometric phenotyping can be used to assist in identifying subtypes of leukemia that have a different prognosis (e.g., T lymphoplasic leukemia (LL)/lymphoma has a worse prognosis in general than B lymphoplasic leukemia/lymphoma) or require an alternate therapeutic regimen (e.g., acute promyelocytic leukemia).

Lymphoblastic Leukemia (LL)/Lymphoma

Immunophenotyping is essential for the diagnosis of LL, and separation of T- and B-LL. B-LL makes up 80% of childhood LL. B-cell lineage is usually defined by the presence of surface CD19 and/or cytoplasmic CD22 expression. Immaturity of the cells is recognized by expression of CD10 and terminal deoxynucleotidyl transferase (TdT), a template-independent DNA polymerase. However, this phenotype overlaps with a significant proportion of normal bone marrow precursors (**hematogones**) (Chapter 38). Therefore, recognition of leukemic blasts requires identifying cells with an abnormal phenotype (Figure 40-4 ■). During normal maturation in the bone marrow, B cells gain and lose antigens in synchrony until they acquire a mature B-cell phenotype (Chapter 8). In contrast, leukemic blasts demonstrate asynchronous expression of antigens (e.g., TdT expression without CD34). In addition, aberrant expression of a myeloid antigen (e.g., CD33 or CD13) is found in 30–50% of cases of B-LL. However, the phenotype of leukemic blasts often resembles a normal stage of maturation (e.g., precursor B cells). Therefore, the presence of genetic abnormalities is a better predictor of prognosis and is used in the current WHO classification scheme.

CASE STUDY *(continued from page 860)*

7. Which of the flow cytometry results presented in this case indicate that this is a malignancy of mature lymphocytes (CLL), not ALL?

T-LL accounts for 15–20% of lymphoblastic neoplasms with a leukemic presentation, but the majority of cases of lymphoblastic lymphoma. Most cases of T-LL express the T-cell antigens CD1, CD2, CD3, CD5, and CD7. Unlike mature T cells, T lymphoblasts often express CD3 only in the cytoplasm, not on the cell surface. In addition, blasts differ from mature T cells in either expressing both CD4 and CD8 (cortical thymocyte phenotype) or lacking both CD4 and CD8. TdT is present in more than 90% of cases, and CD10 can be present. The phenotype of T-LL can be abnormal with loss of one or more of these T-cell antigens. Myeloid markers are aberrantly expressed in 25–30% of pre-T-LL cases. In general, the prognosis for patients with T-LL is worse than for those with precursor B-LL.

☑ CHECKPOINT 40-5

Why is it important to do immunophenotyping in a case of LL?

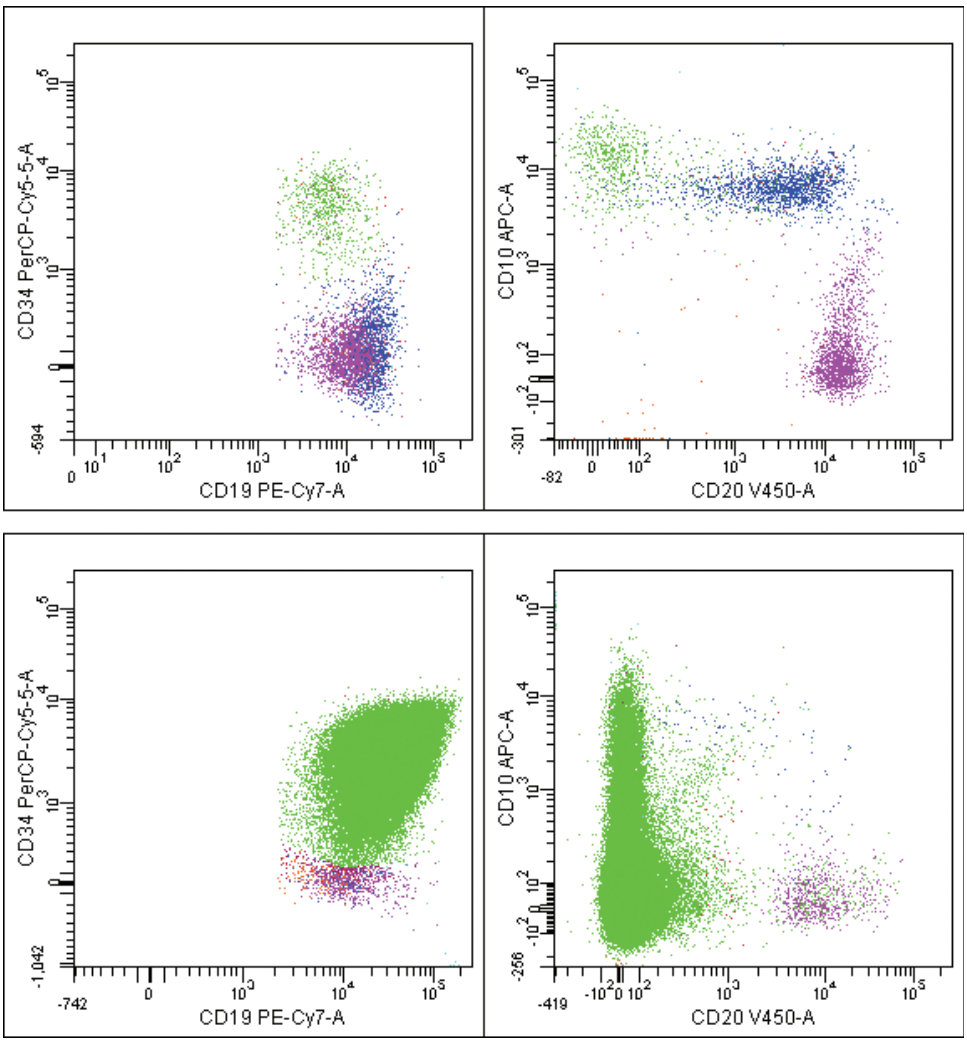


FIGURE 40-4 Aberrant antigen expression in B-LL in comparison with normal precursor B cells. The top two plots display the typical phenotype of normal maturing bone marrow B-cell precursors (hematogones), including a population of most immature cells with expression of CD34 and CD10 but lacking CD20 (green), a population of most mature cells that are CD34−, CD10−, and CD20+ (purple) and an intermediate population with gain of CD20 expression (blue). The lower two plots display an example of B-LL. The leukemic blasts (green) demonstrate CD34 expression and lack of CD20 staining, similar to the most immature normal B cells but demonstrate abnormal, partial CD10 expression. A few mature CD20+, CD10−, CD34− lymphoid cells are also present.

Acute Myeloid Leukemia

The WHO classification scheme recognizes several subtypes of AML (Chapters 23, 26). Most subtypes can be identified using morphology and/or cytogenetics. Immunophenotyping has a limited role in the diagnosis of AML. However, the detection of an abnormal phenotype, such as aberrant expression of a lymphoid antigen, can assist in identifying residual disease following treatment. Immunophenotyping is also required for the diagnosis of AML NOS, minimally differentiated, and is often used for the diagnosis of acute megakaryocytic leukemia. AML NOS, minimally differentiated, is diagnosed when Auer rods are absent and cytochemical stains are negative but there is evidence of nonlymphocytic differentiation by the expression of myeloid antigens. The diagnosis of acute megakaryocytic leukemia relies on identifying megakaryocytic differentiation by immunophenotyping (CD41, CD61) or electron microscopy for recognizing platelet peroxidase positivity with the platelet peroxidase (PPO) stain. Although acute promyelocytic leukemia characteristically lacks staining for CD34 and HLA-DR, a negative CD34 and HLA-DR phenotype is not specific. Therefore, morphology in combination with cytogenetic studies still remains the *gold standard for diagnosing AML*. In addition to diagnosis and classification, flow cytometry can be used to demonstrate the presence of the antigens for directed therapy, for example the presence of CD33 antigen for anti-CD33 monoclonal antibody therapy.

Flow cytometry has also been used to identify abnormalities in the phenotype of myeloid populations in myelodysplastic syndromes.

Abnormalities include aberrant expression of lymphoid antigens on neutrophilic cells and abnormal gain and loss of antigens as cells mature from promyelocytes to segmented neutrophils.

Acute Leukemia of Ambiguous Lineage

Some cases of acute leukemia do not fit neatly into the categories LL and AML. It can be impossible to assign lineage because blasts express antigens from more than one lineage (**mixed phenotype acute leukemia**) or express very few antigens (**acute undifferentiated leukemia**). The WHO classification defines the requirements for assigning each lineage (Table 40-6 ★). By using these criteria, mixed phenotype acute leukemia can be divided into subsets by the lineages represented (e.g., B/myeloid, T/myeloid). However, the phenotype of the blasts and whether the antigens are expressed on the same or separate cells is of less importance in determining the prognosis than the presence of associated genetic abnormalities, such as the *BCR-ABL1* or *MLL* gene rearrangements. The designation acute undifferentiated leukemia requires lack of demonstrable lineage-restricted antigens after comprehensive evaluation. For this evaluation, it is important to include only antigens that have specificity for a lineage. For example, the presence of CD7 alone does not distinguish T-LL from AML because CD7 is normally present on immature T cells and is aberrantly expressed in approximately 10% of AML. In cases of leukemia with a phenotype that does not indicate the lineage, other studies, including morphologic analysis, cytochemical stains, molecular diagnostic studies, and cytogenetic studies, may assist.

Diagnosis and Surveillance of Immunodeficiency Disorders

Immunophenotyping by flow cytometry can be used to analyze leukocyte subsets to identify deficiency of a cell type. The most frequent clinical application of flow cytometry is monitoring the immunodeficiency acquired following HIV virus infection. Less frequently, immunophenotyping is used to detect inherited immunodeficiencies such as the severe combined immunodeficiency disorder (SCID) and X-linked agammaglobulinemia.

The HIV virus uses the CD4 antigen to infect T lymphocytes and monocytes. After viral fusion, internalization, replication, and dissemination, CD4+ cells are destroyed. The resultant decrease in CD4+ cells leads to immunodeficiency. The absolute number and percentage of CD4+ T lymphocytes present in the peripheral blood can be used to monitor the immune system. HIV surveillance is used to predict the disease course, decide when to start prophylactic therapy for opportunistic infections, and determine when to commence antiretroviral therapy. An absolute number of CD4+ T lymphocytes <200/mcL (μL) in the peripheral blood also is used to diagnose AIDS in HIV-infected individuals.

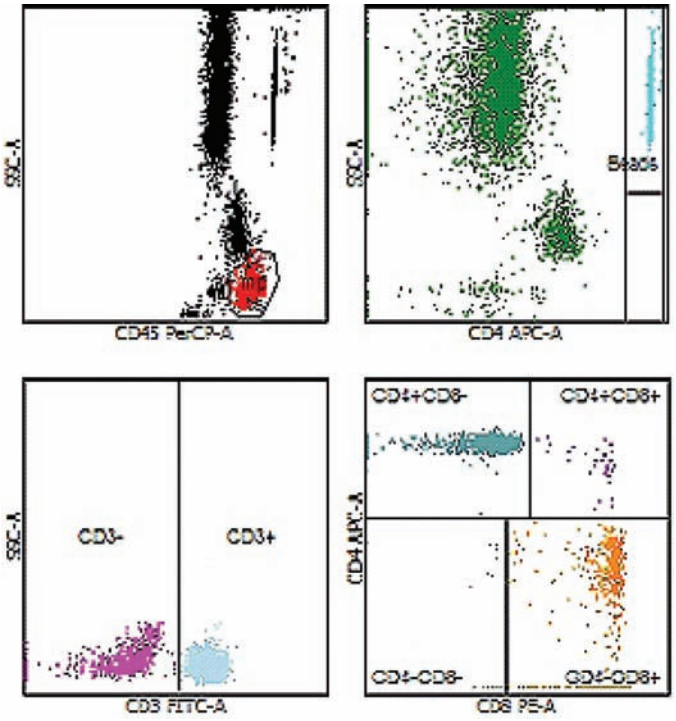
The CD4 count is determined by staining with fluorescent-tagged antibodies against CD3 (found on T cells but not monocytes) and CD4 followed by whole blood RBC lysis. The percentage of CD4+ lymphocytes is determined by flow cytometry. Gating is critical to avoid exclusion of lymphocytes and contamination by cells other than lymphocytes such as CD4+ monocytes. The absolute CD4 count can be determined directly by flow cytometry (single platform) by comparing the number of CD4+ cells identified per unit volume analyzed with an absolute number of highly fluorescent beads added to the analysis tube (Figure 40-5 ■). Alternatively, the CD4 count can be calculated from the percentage of CD4+ lymphocytes and the absolute number of lymphocytes determined by a hematology analyzer (dual platform):

$$\text{Absolute CD4 count} = \text{Absolute lymphocyte count} \times \%\text{CD4+ Lymphocytes}/100$$

$$\text{Absolute lymphocyte count} = \text{WBC count} (\times 10^9/\text{L}) \times \%\text{Lymphocytes from WBC differential}/100$$

★ **TABLE 40-6** Flow Cytometric Requirements for Assigning Blast Lineage

Lineage	Requirement
Myeloid	Myeloperoxidase or monocytic differentiation (CD11c, CD14, CD64)
T cell	CD3 cytoplasmic (epsilon chain) or CD3 surface
B cell	Strong CD19 along with at least one other B-cell marker (CD79a, CD22 cytoplasmic, or CD10) or weak CD19 along with at least 2 B-cell markers (CD79a, CD22 cytoplasmic, or CD10)



■ **FIGURE 40-5** CD4 enumeration. CD4+ lymphocytes are isolated by gating on cells with bright intensity CD45 and low side light scatter (red, top left dot plot), bright intensity CD3 (blue, lower left dot plot), and CD4 (greenish-blue, lower right dot plot). The absolute CD4 count is determined by comparing the number of CD4+ events with the number of beads counted (top right dot plot).

Analysis performed using BD Multitest™, BD Biosciences, San Jose, CA

A precise and accurate CD4 count is essential (Table 40-7 ★). Results from a patient are often compared with previous results and published diagnostic and therapeutic levels. Therefore, the procedure used for quantitative flow cytometry should attempt to eliminate preanalytical and analytical variables. Preanalytical variables include biologic variability (age, diurnal rhythm, and medications), specimen collection, and storage. Analytical variables include sample preparation and errors associated with flow cytometers and hematology analyzers. The CDC publishes guidelines for standardization of CD4 determination. Two levels of stabilized quality control material must be run with each batch of CD4 assays. In addition, proficiency testing for professionals is available through College of American Pathologists (CAP) surveys.

The HIV viral load is another monitor of HIV infection. Viral load testing often measures viral RNA in plasma. The CD4 count and the HIV viral load often provide complementary information. The CD4 count is the best indicator of the balance between immune cell production and destruction and therefore indicates the risk of opportunistic infection or secondary malignancy. The HIV viral load indicates the burden of disease and therefore can be used to monitor response to antiviral therapy and the acquisition of drug resistance.

☑ CHECKPOINT 40-6

A patient has 10% CD4+ lymphocytes, a WBC count of $5 \times 10^9/L$, and 30% lymphocytes. What is the absolute CD4 count? Is this count compatible with a diagnosis of AIDS in an HIV-infected individual?

CD34 Enumeration

CD34 enumeration is used to support bone marrow and peripheral blood stem cell transplantation (Chapter 29). CD34 is an antigen restricted to multipotential hematopoietic stem cells and early progenitors of all lineages. Hematopoietic stem cells are responsible for reconstituting bone marrow function following transplantation. CD34 enumeration is used to determine whether the product collected for transplantation has enough cells for rapid and long-term engraftment. CD34+ cells can be collected by either bone marrow aspiration or peripheral blood apheresis. Hematopoietic progenitor cells are often mobilized into the peripheral blood using a combination of cytotoxic drugs and growth factors. CD34 enumeration can also be performed on the peripheral blood to determine the optimal time for peripheral blood stem cell collection. CD34 determination is challenging because it needs to be fast, accurate, and precise even at low numbers (0.1% of cells or 5 cells/mcL [μL]). Significant progress has been made in standardizing CD34 flow cytometric assay procedures and analysis protocols (Table 40-8 ★).[5,6]

★ **TABLE 40-7** Procedural Requirements for Quantitative Flow Cytometry

Standardization of procedure
• Patient preparation
• Specimen handling
• Specimen preparation
• Analysis (gating accuracy and purity and precision)
Quality control
Proficiency testing
Documentation of personnel competency

★ **TABLE 40-8** Recommendations for CD34 Enumeration

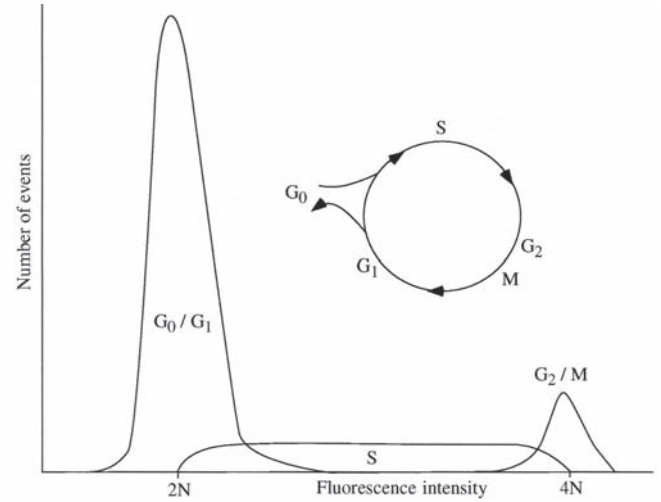
Use bright (e.g., PE) fluorochrome conjugates of class II or III monoclonal antibodies that detect all glycoforms of CD34.
Use a vital nuclei acid dye to exclude platelets, unlysed RBCs, and debris or 7-amino actinomycin (7-AAD) to exclude dead cells during acquisition.
Include CD45 to assist in detection of hematopoietic progenitor cells.
Use a Boolean gating strategy to resolve the CD34+ HPC from irrelevant cells.
Include the identification of cells with low levels of CD45 expression and low side scatter and include CD34 bright and dim staining populations.
Acquire sufficient events to generate at least 100 CD34+ cells to ensure a 10% precision.

Paroxysmal Nocturnal Hemoglobinuria (PNH)

PNH is a chronic hemolytic disorder caused by an acquired mutation of the *PIG-A* gene (Chapter 17), which encodes an enzyme critical in the synthesis of the glycosylphosphatidylinositol (GPI) anchor that links many proteins to the cell membrane. Flow cytometry can be used to detect the presence of GPI-anchored membrane proteins. In addition, some flow cytometric assays assess the ability of white blood cells to bind a fluorescent-labeled modified toxin (FLAER) that attaches through GPI anchors. Flow cytometric immunophenotyping of erythrocytes and leukocytes is a more sensitive and specific test for diagnosing PNH than the sucrose hemolysis test or Ham test. Erythrocyte immunophenotyping is easier to interpret than analysis of neutrophils but can give a false negative result if hemolysis is active or a transfusion was recently given. Cells with a PNH-type phenotype have also been identified in patients with aplastic anemia and some myelodysplastic syndromes and in very small numbers in normal individuals.[7]

DNA ANALYSIS

DNA analysis is usually performed on solid tumors to provide prognostic information. Either fresh frozen tissue or a thick section of fixed, paraffin-embedded tissue is processed to produce individual cells or nuclei. The suspension is permeabilized (the cell and/or nuclear membrane is made permeable to allow access of the fluorescent dye to the DNA) and stained with a fluorochrome that binds to DNA. If the fluorescent dye (e.g., propidium iodide) binds to both DNA and RNA, RNA is removed by digestion. The amount of fluorochrome bound is proportional to the amount of DNA. The DNA content varies during cell division (proliferation) and is abnormal in cells with numerical chromosome abnormalities (aneuploid) (Figure 40-6 ■). Computer



■ **FIGURE 40-6** Fluorescence intensity of DNA-bound dye during phases of the cell cycle. Mathematical programs are used to distinguish three areas under the curve (cell-cycle phases G_0/G_1,S, and G_2/M).

software uses mathematical formulas to calculate the cells in each phase of the cell cycle, calculate indexes, and identify abnormal populations.

Proliferation

Tumors with an increased number of dividing cells often have a worse prognosis but in some instances are susceptible to therapy directed at dividing cells. The DNA content of cells can be used to determine the proportion of dividing cells. During cell division and prior to mitosis, the DNA content increases (synthesis, or S phase) until there are two copies of the entire genome prior to mitosis (G_2/M-phase) (Figure 40-6; Chapter 2). The *S phase fraction* is the proportion of all cells (G_0/G_1 + S + G_2/M) that are in the S phase. The *proliferative index* is the proportion of all cells that are in proliferative phases (S + G_2/M) (Table 40-9 ★).

★ **TABLE 40-9** Calculations for DNA Analysis by Flow Cytometry

S phase fraction	SPF = 100 × S/(G_0/G_1 + S + G_2/M)
Proliferation index	PI = 100 × (S + G_2/M)/(G_0/G_1 + S + G_2/M)
DNA index	DNA index = DNA content G_0/G_1 sample/ DNA content G_0/G_1 diploid control

Ploidy

All nondividing, normal human cells contain 46 chromosomes (2 each of 22 autosomes, and the sex chromosomes, either XX or XY) and are therefore referred to as being *diploid*. The presence of tumor cells containing an abnormal number of chromosomes is often associated with a worse prognosis. The **DNA Index (DI)** is the DNA content of tumor cells relative to a diploid population of cells. It is calculated as the DNA content of cells in the tumor in the G_0/G_1 phase of the cell cycle relative to the DNA content of G_0/G_1 cells in a diploid control. For example, diploid cells have a DI of 1.0. Tetraploid cells (four copies of all chromosomes) have a DI of 2.0, and cells with only one copy of each chromosome (haploid) have a DI of 0.5. *Aneuploidy* is the presence of cells with an abnormal DNA content that is not a multiple of the DNA content of haploid cells, for example, a DI of 1.3.

Clinical Applications of DNA Analysis

The clinical utility of DNA analysis remains controversial. One of the most extensively studied tumors is breast carcinoma. A correlation exists between increased S phase fraction and other markers of a poor prognosis (large breast tumor, the presence of metastatic disease in axillary lymph nodes, high histologic grade, and absence of steroid receptors). However, the main value of DNA analysis in breast carcinoma can be the identification of patients who have a low S phase fraction and might not require chemotherapy in addition to surgical excision. Interlaboratory variability in methodology and interpretation of results has delayed the routine use of this technique.[8]

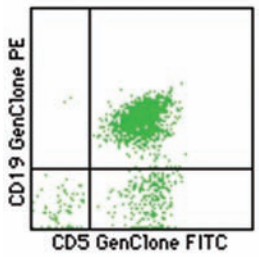
Summary

Flow cytometry is a technique that involves the analysis of single particles for their ability to scatter light and fluoresce. This technology is used by stand-alone instruments (flow cytometers) and has also been incorporated into other instruments such as hematology cell analyzers. Flow cytometry is used in the clinical laboratory for immunophenotyping, reticulocyte counting (discussed in Chapter 11 and Chapter 39), and DNA analysis. Immunophenotyping uses fluorochrome-labeled detection antibodies to identify cellular antigens. Its primary use is to classify and subtype leukemia and lymphoma. Reticulocyte and DNA analysis utilize fluorescent dyes that bind to nucleotides. DNA analysis is used to analyze solid tumors.

Review Questions

Level I

1. Which of the following is a (are) component(s) of a flow cytometer? (Objective 1)
   A. flow chamber
   B. laser light source
   C. light detectors
   D. all of the above
2. Which of the following properties of the cells analyzed by flow cytometry is related to forward angle light scatter? (Objectives 1, 7)
   A. granularity
   B. nuclear complexity
   C. size
   D. shape
3. Which of the following statements explains the use of fluorochromes in immunophenotyping by clinical flow cytometry? (Objectives 3, 5)
   A. All fluorochromes emit light at the same wavelength.
   B. Fluorochromes bind nonspecifically to leukocytes.
   C. The wavelength of the emitted light is the same as that of the incident light.
   D. Several fluorochromes can be excited by light of a single wavelength.
4. Which of the following properties is (are) used for gating in clinical flow cytometry? (Objective 1)
   A. forward angle light scatter
   B. side angle light scatter
   C. intensity of CD45 staining
   D. all of the above
5. Which of the following is a (are) clinical application(s) of flow cytometry? (Objective 2)
   A. DNA quantitation
   B. immunophenotyping
   C. leukemia classification
   D. all of the above
6. Which of the following flow cytometry methods is the most appropriate for diagnosing and classifying chronic lymphocytic leukemia? (Objectives 4, 5, 7)
   A. CD34 enumeration
   B. DNA quantitation
   C. immunophenotyping
   D. RNA quantitation
7. Which of the following is detected during immunophenotyping by flow cytometry? (Objective 3)
   A. antibodies using detection antigens
   B. antibodies using detection fluorochromes
   C. fluorochromes using detection antibodies
   D. antigens using detection antibodies
8. Which of the following specimens is most appropriate for flow cytometry immunophenotyping? (Objective 6)
   A. formalin-fixed bone marrow aspirate clot
   B. fresh lymph node biopsy
   C. frozen bone marrow biopsy
   D. one-week-old (ethylenediaminetetraacetic) EDTA anticoagulated peripheral blood
9. A peripheral blood sample needs to be shipped to a reference lab for flow cytometry immunophenotyping. Which of the following procedures is most appropriate? (Objective 6)
   A. overnight delivery at room temperature
   B. overnight delivery at 4°C
   C. regular mail service at room temperature
   D. regular mail service at 4°C
10. An 80-year-old male was found to have lymphocytosis. Flow cytometry of the peripheral blood revealed the following results:

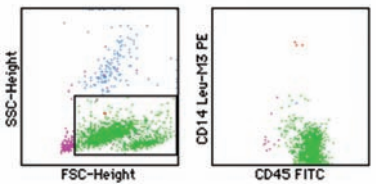


What is the phenotype of the majority of lymphocytes represented in this diagram? (Objective 4)

A. CD19+, CD5−
B. CD19+, CD5+
C. CD19−, CD5−
D. CD19−, CD5+

Level II

1. A 45-year-old female presented with pancytopenia. Flow cytometry performed on the peripheral blood revealed the following results:



Which is the predominant cell type in the gate? (Objective 9)

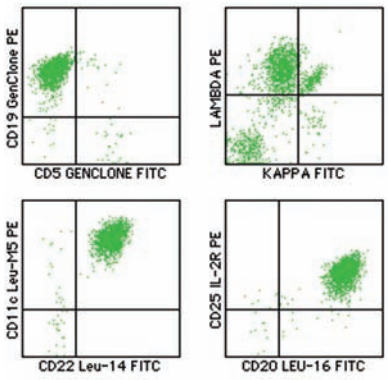
A. eosinophil

B. lymphocyte

C. monocyte

D. neutrophil

2. Further flow cytometric analysis performed on the sample described in question 1 revealed the following results:



What is the phenotype of the cells present? (Objective 9)

A. CD19+, CD5−, CD11+, CD25+

B. CD19+, CD5+, CD11+, CD25+

C. CD19+, CD5−, CD11c−, CD25−

D. CD19−, CD5−, CD11c−, CD25−

3. Which of the following phenotypes is indicated by the κ and λ light chain dot plot displayed in question 2? (Objective 9)

A. absence of surface immunoglobulin

B. monoclonal λ immunoglobulin light chain

C. monoclonal κ immunoglobulin light chain

D. polyclonal immunoglobulin light chains

4. Which of the antigens expressed in question 2 indicates a mature B lymphocyte phenotype? (Objective 1)

A. CD5

B. CD11c

C. CD19

D. surface immunoglobulin

5. Which of the following is the most likely diagnosis for the case described in question 2? (Objective 1)

A. ALL

B. chronic lymphocytic leukemia

C. hairy cell leukemia

D. infectious mononucleosis

6. Flow cytometric analysis of a lymph node fails to reveal an abnormal phenotype. The pathologist is convinced that the node is involved by lymphoma. A touch preparation performed from the specimen received in the laboratory contains numerous large cells that are absent from a cytocentrifuge slide made from the cell suspension prepared for staining. Which of the following is the most likely explanation? (Objective 4)

A. The sample received for flow cytometry does not represent the malignancy.

B. The malignant cells were lost during manual disaggregation of cells.

C. The malignant cells were excluded from the gate.

D. The malignant cells have a normal phenotype.

7. Repeat analysis of the case described in question 6 increases the number of large cells present in the suspension stained and analyzed on the flow cytometer. An abnormal phenotype is still not detected. Which of the following neoplasms could be difficult to detect by flow cytometry cell surface antigen studies? (Objective 2)

A. Hodgkin's disease

B. T-cell lymphoma

C. plasma cell neoplasms

D. all of the above

8. A patient presented with circulating blasts. Flow cytometry immunophenotyping performed on the peripheral blood revealed the following phenotype: CD19+, CD10+, surface immunoglobulin IgM κ. Which of the following is the most likely diagnosis? (Objective 3)

A. Burkitt lymphoma

B. acute LL, precursor B-cell type

C. acute LL, precursor T-cell type

D. AML

9. Pitfalls in diagnosing mature lymphoid malignancies by flow cytometry include: (Objective 2)
   A. Malignant B cells can lack surface immunoglobulin.
   B. Some malignant lymphoid cells lack diagnostic surface antigens.
   C. Neoplastic cells can be rare.
   D. All the above

10. A patient was found to have circulating blasts. No Auer rods were identified, and all cytochemical stains were negative. Flow cytometry immunophenotyping revealed the presence of myeloid antigens. Which of the following is the most appropriate diagnosis? (Objective 3)
   A. acute LL
   B. AML
   C. chronic lymphocytic leukemia
   D. chronic myeloid leukemia

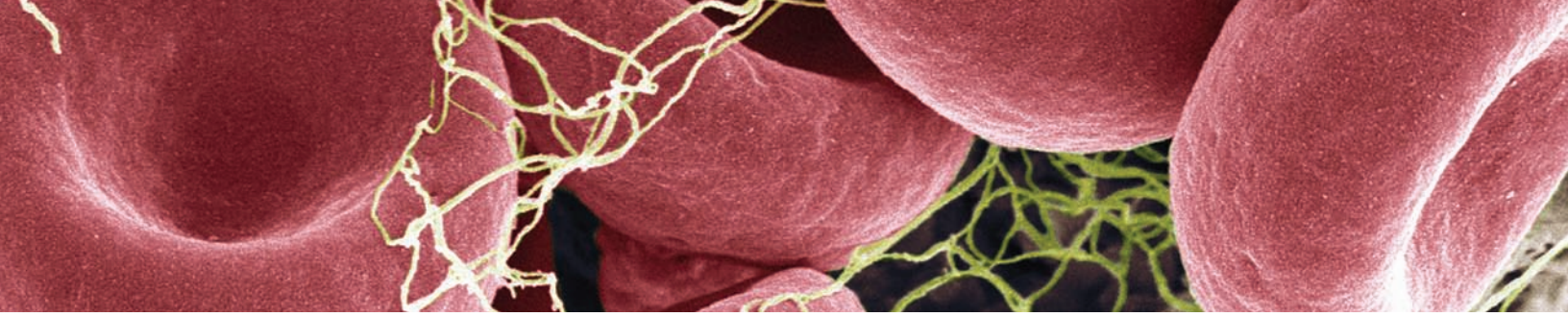
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Givan AL. Principles of flow cytometry: an overview. *Methods Cell Biol.* 2001;63:19–50.
2. Swerdlow SH, Campo E, Harris NL et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th ed. Lyon, France: IARC Press; 2008.
3. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood.* 2008;111:3941–967.
4. Chen YH, Peterson LC, Dittman D et al. Comparative analysis of flow cytometric techniques in assessment of zap-70 expression in relation to IgVh mutational status in chronic lymphocytic leukemia. *Am J Clin Pathol.* 2007;127:176–81.
5. Gratama JW, Orfao A, Barnett D et al. Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells. European Working Group on Clinical Cell Analysis. *Cytometry.* 1998;34:128–42.
6. Whitby A, Whitby L, Fletcher M et al. ISHAGE protocol: are we doing it correctly? *Cytometry B Clin Cytom* 2012:82:9–17.
7. Borowitz MJ, Craig FE, DiGiuseppe JA et al. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. *Cytometry B Clin Cytom;* 2010:78:211–30.
8. Wenger CR, Clark GM. S-phase fraction and breast cancer–a decade of experience. *Breast Cancer Res Treat.* 1998;51:255–65.



41 Chromosome Analysis of Hematopoietic and Lymphoid Disorders

KATHLEEN S. WILSON, MD, FCAP, FACMG

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Define *chromosome* and *mitosis*.
2. List the basic steps of cytogenetic analysis, and select the most appropriate type of specimen for analysis of suspected constitutional and neoplastic (acquired) disorders.
3. Identify the major types of chromosome abnormalities, describe how they occur, and use the appropriate terminology to describe them.
4. List the practical uses of cytogenetics in the diagnosis and prognosis of hematolymphoid disorders.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Describe chromosome morphology and mitosis.
2. Determine which specimen type is most appropriate for various clinical indications.
3. Describe each step of the cytogenetic harvest and banding procedure.
4. Define and compare aneuploidy, nondisjunction, and anaphase lag.
5. Define and compare translocation, deletion, inversion, and isochromosome.
6. Identify the general categories of hematopoietic disorders for which cytogenetic analysis is useful for patient care.
7. Correlate diagnostic chromosome aberrations with types of hematolymphoid disorders, and assess their prognostic and therapeutic implications.
8. Assess the prognostic impact of cytogenetic results in lymphoblastic leukemia (acute lymphoblastic leukemia, ALL).
9. Explain the clinical utility of cytogenetics in transplantation.
10. Correlate chromosome abnormalities with specific oncogene activation, and assess the role of the oncogene in the neoplasm.

Chapter Outline

Key Terms

Acquired aberration
Acrocentric
Aneuploid
Chimerism
Constitutional cytogenetic aberration
Cytogenomic microarray analysis (CMA)
Diploid
Endomitosis
Haploid
Heterologous
Homologous
Hyperdiploid
Hypodiploid
Karyotype
Metacentric
Monosomy
Mosaic
Nondisjunction
Polymorphic variant
Polyploid
Pseudodiploid
Satellite DNA
Submetacentric
Trisomy

Background Basics

The information in this chapter builds on the concepts presented in other chapters relating to cell division (Chapters 2, 4) and hematopoietic and lymphoid neoplasms (Chapters 23–28). To maximize your learning, you should review these concepts before beginning this unit of study:

Level I

- Describe the stages of the cell cycle, particularly the steps of mitosis. (Chapter 2)
- List the major types of neoplastic hematopoietic and lymphoid neoplasms. (Chapter 23)

Level II

- Outline the classification of acute myeloid leukemia and acute lymphoblastic leukemia. (Chapters 26, 27)
- Describe the various typical laboratory findings and criteria for classification of myeloproliferative neoplasms. (Chapter 24)
- Describe the typical laboratory findings and classification of the myelodysplastic states. (Chapter 25)
- Summarize the chronic lymphoproliferative neoplasms, list the criteria for distinction of Hodgkin's disease versus non-Hodgkin lymphomas, and define the major classification terminology. (Chapter 28)

CASE STUDY

We will address this case study throughout the chapter.

Gregory, a healthy 25-year-old man, has a routine physical examination and laboratory studies for new employment. The total white blood cell (WBC) count is 30,000 $\times$ 10^9/L.

Consider what conditions could result in this clinical picture and the follow-up studies that should be done.

OVERVIEW

This chapter is designed to give the reader a background for understanding the terminology and application of cytogenetics in the diagnosis and treatment of *hematolymphoid neoplasms* (a term commonly used to encompass both hematopoietic and lymphoid neoplasms). The chapter begins with a review of chromosome structure and morphology and is followed by a summary of the procedure used to prepare specimens for chromosome studies. Chromosomal abnormalities are discussed, and the terminology used to describe cytogenetic findings is defined. The remainder of the chapter discusses practical uses of cytogenetic analysis of hematolymphoid neoplasms.

INTRODUCTION

Cytogenetics is the study of chromosome structure and number, particularly as they relate to a normal or pathologic state. In 1956, the normal number of chromosomes per human cell was established as 46, and since that time, many chromosome abnormality syndromes have been reported. The use of cytogenetics has markedly improved patient diagnosis and family counseling in the field of constitutional aberrations, including important advances in prenatal diagnosis. Cytogenetic studies are also responsible for major advances in the field of hematolymphoid malignancies and solid tumors. Chromosome analysis of many malignant disorders has become a critical component for patient diagnosis and prognosis as well as for research studies of these disorders.

CHROMOSOME STRUCTURE AND MORPHOLOGY

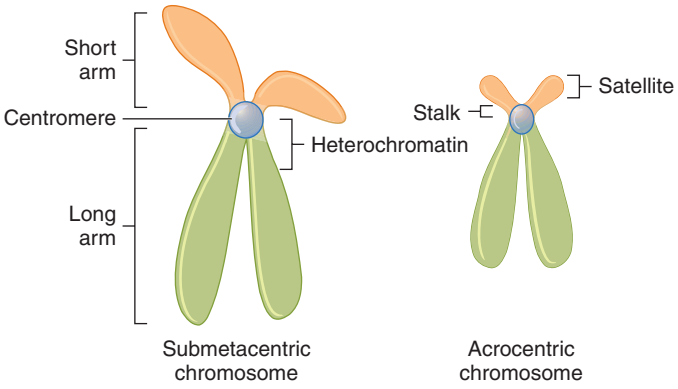
Nuclear chromatin of human cells is composed of nucleic acid and protein and is organized into 46 chromosomes. The nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are composed of polynucleotides. A single nucleotide consists of a phosphate, a sugar (deoxyribose for DNA and ribose for RNA), and a base. The base can be a purine (A = adenine, G = guanine) or a pyrimidine (C = cytosine and T = thymine [DNA] or U = uracil [RNA]). The bases are aligned on the polynucleotide strand in a triplet code so that three bases code for a single amino acid; the succession of bases in the triplet code determines the protein products that will result from transcription of the DNA and translation of the messenger RNA produced. Human cells have approximately 20,000 genes in each located at a specific site on a specific chromosome (the gene locus). The different possible expressions of a gene are known as *alleles*. For example, the gene for the ABO blood group has three major alleles: A, B, and O.

DNA exists as a double-stranded helix with the two polynucleotide strands held together by hydrogen bonds between complementary

bases so that G will bind only with C and A only with T (or U for RNA). The bonding of A-T and G-C is called a *base pair*. This double helix has a diameter of approximately 20 Å and is of variable length. For example, the amount of DNA contained in the smallest chromosome, number 21, is composed of approximately 50 million base pairs, whereas the largest chromosome, number 1, has approximately 250 million base pairs. The double helix coils around histone proteins resulting in a series of structures called *nucleosomes* (Figure 41-1 ■). These nucleosomes form a superhelix with six nucleosomes per turn forming a chromatin fiber (called a *solenoid*) with a diameter of 250 Å. These fibers are looped back and forth on a protein-RNA scaffold to form an identifiable chromatid with a diameter of 0.2– 0.5 mcM (μm) After DNA replication, identical sister chromatids are connected at the centromere, giving the final structure of a mitotic chromosome.

The centromere divides chromosomes into short (p) arms and long (q) arms (Figure 41-2 ■). If a centromere is in the center of the chromosome so that the length of p = q, it is referred to as a **metacentric** chromosome (chromosomes 1, 3, 16, 19, and 20). If the centromere is not in the center, so that p < q, the chromosome is called **submetacentric** (chromosomes 2, 4, 5, 6–12, 17, 18, and X), and when the centromere is located close to the end of the chromosome so that p is very short, the chromosome is called **acrocentric** (chromosomes 13–15, 21, 22, and Y). The area of the chromosome around the centromere contains highly repetitive DNA in long clusters of tandem repeats, also known as **satellite DNA**, which is permanently coiled tightly into heterochromatin. Heterochromatin stains darkly and is transcriptionally inactive; euchromatic areas stain lighter and are transcriptionally active during interphase. The heterochromatin on the end of the p arm of acrocentric chromosomes is attached to the rest of the p arm by less tightly coiled chromatin stalks (Figure 41-2).

A normal human cell has 46 chromosomes consisting of 23 pairs. Chromosome pairs 1–22 are autosomes, and the X and Y chromosomes are sex chromosomes. Before banding techniques were developed, the chromosome numbers were assigned according to the total length of the chromosome beginning with number 1 as the longest and number 22 as the shortest. With the advent of chromosome banding,

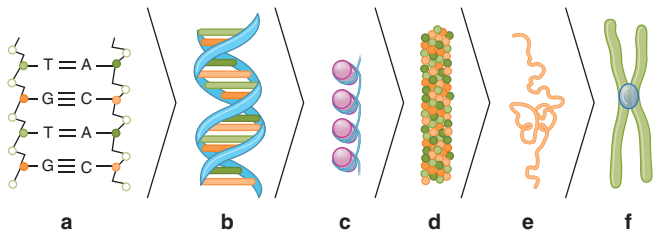


■ **FIGURE 41-2** Chromosome structure of a typical submetacentric chromosome and an acrocentric chromosome. The short arm is known as "p" and the long arm as "q".

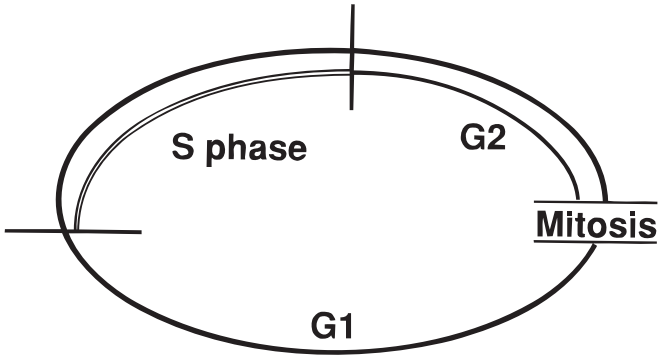
chromosome 21 was determined to be shorter than 22, but the designations were not changed. A **homologous** chromosome pair consists of two morphologically identical chromosomes that have identical gene loci but can have different alleles at a given locus because one member of a homologous pair is of maternal origin and the other is of paternal origin. For example, a homologous pair consisting of both chromosomes number 9 has the gene locus for the ABO blood group on the long arm. An individual can inherit the allele for blood group A on the maternal number 9 chromosome and the allele for blood group B on the paternal number 9 chromosome. A **heterologous** pair (i.e., the sex chromosomes, X and Y, in a male) consists of morphologically nonidentical chromosomes that have different gene loci.

MITOSIS

Cells that go through a proliferative division do so by a series of stages called the *cell cycle*; it consists of four major phases: G_1, S, G_2, and M (mitosis) (Figure 41-3 ■) (Chapter 2). A cell that is not dividing but is performing its designated function is in interphase. Interphase begins



■ **FIGURE 41-1** Chromosome morphology and ultrastructure. **(a)** Molecular structure of DNA with two polynucleotide chains held together by hydrogen bonding of base pairs (T = thymine, C = cytosine, A = adenine, G = guanine) **(b)** Double helical structure of DNA. **(c)** Coiling of double helix strand around histone proteins to produce nucleosome. **(d)** Superhelix of nucleosome producing chromatin fiber. **(e)** Coiling of chromatin fiber to produce chromomere. **(f)** Final structure of chromosome consisting of two identical sister chromatids (condensed chromomere) held together at the centromere.



■ **FIGURE 41-3** Diagram of the cell cycle beginning with G_1, the stage in which the cell is performing its designated duties. The DNA is uncoiled and exists as 46 single chromatids. S phase is the time of DNA synthesis after which the DNA is still uncoiled and consists of 46 chromosomes (sister chromatids joined at the centromere). G_2 is a resting phase followed by mitosis.

at G_1 of the cell cycle and continues until the end of G_2. During G_1, the nuclear chromatin is dispersed, and chromosome morphology is not identifiable. The next phase, S, is the time of DNA synthesis. The DNA is replicated, and identical sister chromatids are attached at the centromere. The S phase is followed by a short resting phase, G_2, after which the cell enters mitosis. The length of time that a cell spends in each phase is quite variable. The average time that a cell spends in mitosis is estimated to be ~45 minutes.

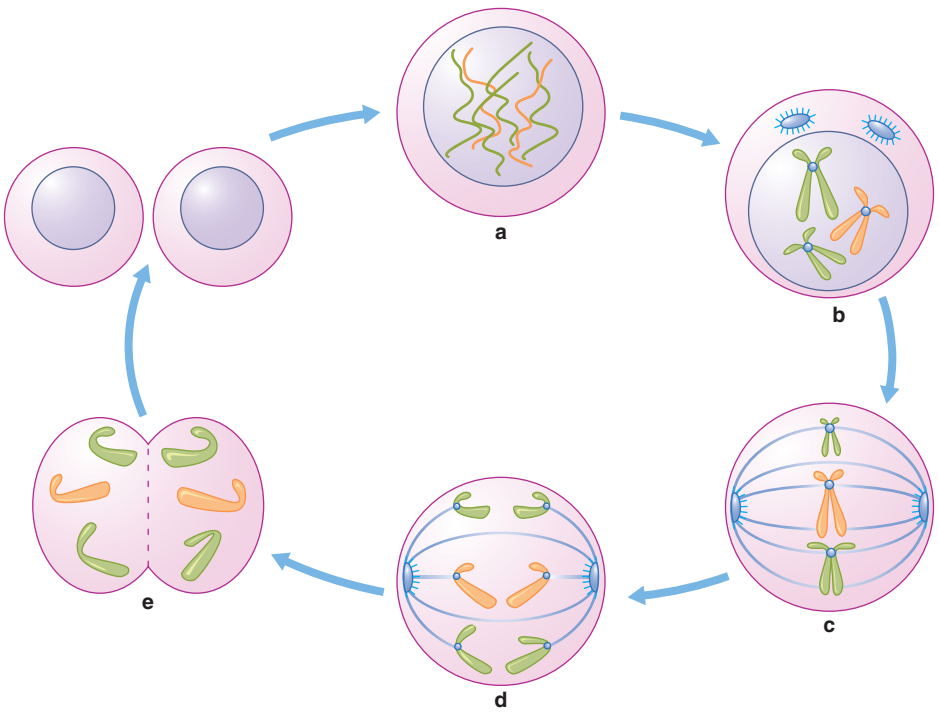
To study human chromosomes, the cells must be mitotically active. *Mitosis* is the process of division of somatic cells by which each daughter cell ends with the same genetic composition as the parent cell. *Prophase* is the first stage of mitosis (Figure 41-4 ■) during which the DNA begins to coil, chromosome morphology becomes recognizable, and a pair of cytoplasmic organelles known as *centrioles*, which are attached to the mitotic spindle, migrate to opposite poles of the cell. In metaphase, the DNA is tightly coiled and the chromosomes align in the center of the cell (equatorial plate, metaphase plate), and the mitotic spindle apparatus attaches to the kinetochores of the chromosome centromere via microtubules. *Anaphase* begins with the contraction of the spindle fibers pulling apart the sister chromatids so that one sister chromatid migrates to one pole of the cell and the other migrates to the opposite pole of the cell. During *telophase*, the daughter nuclei begin to form at the opposite poles of the cell, and cytokinesis begins. At the end of telophase, *cytokinesis* (the division of the cytoplasm into two daughter cells) completes, and each of the daughter cells then reenters the G_1 phase of the cell cycle. Each resultant daughter cell has the identical genetic composition as the parent cell.

Meiosis is the specialized division of diploid (46 chromosomes) primary gametocytes that results in each gamete (oocyte and sperm) having a haploid number of chromosomes (23). The understanding of meiosis is critical for the study of constitutional chromosome aberrations and is not discussed in this chapter.

CYTOGENETIC PROCEDURES

Specimen Preparation

Specimens submitted for cytogenetic analysis must have viable cells capable of undergoing mitosis; the choice of appropriate specimens depends on the patient's clinical situation (Table 41-1 ★). For the evaluation of possible **constitutional cytogenetic aberrations** (aberrations present in every cell in a patient's body, i.e., its constitution), peripheral blood is the most appropriate sample because circulating lymphocytes can be easily manipulated to undergo mitosis by the use of mitogens. For example, phytohemagglutinin (PHA) stimulates predominantly T lymphocytes. If the patient has a lymphocytopenia or if there is a suspicion that cells from different tissue types can have different karyotypes (a condition termed *mosaicism*), a punch-biopsy of skin can be obtained for fibroblast culture. When the clinical situation involves a spontaneous abortion (miscarriage), stillbirth, or death shortly after birth, the appropriate sample is fetal tissue (products of conception) or autopsy-acquired organ samples (lung, liver, kidney, diaphragm). A prenatal evaluation requires culturing either amniotic fluid cells or a chorionic villus biopsy processed by direct harvest and tissue culture. These various cultures are harvested at the time of



■ **FIGURE 41-4** Mitosis. **(a)** Interphase. Chromatin is dispersed. **(b)** Prophase. Chromosome structure is discernible and centrioles begin to migrate. **(c)** Metaphase. Chromosomes are lined up in the center, spindle fibers from the centrioles connect to the centromeres, and the nuclear membrane is not visible. **(d)** Anaphase. Spindle fibers contract and sister chromatids migrate to opposite poles of the cell. **(e)** Telophase. Chromatid migration is complete and the cytoplasmic membrane forms down the center, completing the cell division.

★ **TABLE 41-1** Appropriate Specimens for Cytogenetic Analysis

Clinical Situation	Appropriate Specimen	Type of Processing
Constitutional aberrations	Peripheral blood	Mitogen stimulation of lymphocytes with phytohemagglutinin
	Skin biopsy	Tissue culture
	Autopsy organ samples	Tissue culture
	Products of conception	Tissue culture
	Amniotic fluid	Tissue culture
	Chorionic villus sample	Direct harvest, tissue culture
Neoplastic (acquired) aberrations	Peripheral blood/bone marrow	Direct harvest, unstimulated cultures; stimulated cultures of mature B-cell malignancies; all cultures from cell suspension
	Lymph node/spleen	Direct harvest, unstimulated cultures; stimulated cultures of mature B-cell malignancies; all cultures from cell suspension
	Solid tumor biopsy	Monolayer and suspension tissue culture

maximal mitotic activity, usually 72–96 hours for peripheral blood and 7–21 days for amniotic fluid and fibroblast cultures.

Acquired aberrations (those happening after birth in a single cell) occur in neoplastic processes such as leukemia, lymphoma, and other tumors. Evaluation of these disorders requires that the neoplastic cells be sampled directly either by peripheral blood, bone marrow, or solid tumor biopsies. These samples are harvested immediately (direct harvest) or by short-term unstimulated cultures to obtain mitoses of the neoplastic cells, not the associated nontumor cells.

The first step of the cytogenetic procedure is to induce cells into mitosis. Once this has been accomplished by any of the methods named, the cells are "harvested." The harvest procedure processes cells that are mitotically active to visualize the chromosomes. The major steps are metaphase inhibition, hypotonic incubation, and fixation.

CASE STUDY *(continued from page 870)*

The differential WBC count shows 7% blasts, 3% promyelocytes, 25% myelocytes, 10% metamyelocytes, 5% bands, 25% segmented neutrophils, 10% basophils, 5% eosinophils, and 10% lymphocytes. The differential diagnosis includes leukemoid reaction, chronic myeloid leukemia (CML), and myeloproliferative neoplasms other than CML. A bone marrow aspirate is performed.

1. What is the most appropriate specimen to submit for cytogenetics, and how should it be processed?

Harvest Procedure and Banding

Mitotically active cells are stopped in metaphase by incubation with agents that disrupt the spindle apparatus, most commonly colchicine or colcemid.[1] The cells are then incubated with a hypotonic solution (frequently 0.075 M KCl) that hemolyzes erythrocytes and partially swells the nucleated cells. Fixation of the cells is then accomplished with Carnoy's fixative, 3:1 methanol:glacial acetic acid. After fixation, a trial slide is prepared by putting three to four drops of the final cell suspension onto a clean glass slide. The slide is dried and examined by phase microscopy for appropriate spreading and number of mitotic figures. If the first slide does not show optimal quality, the suspension can be concentrated or diluted, or other manipulations can be done to obtain improved chromosome morphology. The remainder of the slides are then prepared and "aged" for banding; this often involves heating the slides in a 60°C oven overnight.

Chromosome banding is obtained by various staining procedures that result in a specific pattern of dark-to-light stained bands for each homologous chromosome pair. The first chromosome banding technique was reported in 1970 with the use of quinacrine (Q), a fluorescent stain that reveals a pattern of bright and dull bands (Q-bands).[2] Q-banding techniques are relatively simple; however, the banding fades when examined microscopically with ultraviolet illumination, and the resolution of bands is not as detailed as are G-bands (G = Giemsa). Most laboratories routinely analyze G-bands using Giemsa stain and some form of enzyme pretreatment, usually trypsin.[3] These techniques result in a high-quality banding pattern that does not fade with microscopic examination. The pattern of bright or dull Q-bands and dark- or pale-staining G-bands is essentially the same. Reverse banding (R-bands) yields a band pattern opposite to that of Q- and G-bands so that a pale G-band will be darkly stained with R-banding. Other banding techniques and special harvest procedures can be helpful for evaluation in certain clinical situations (Table 41-2 ★).

The development of fluorescently labeled DNA probes for specific chromosome centromeres, whole arms, whole chromosomes, and individual genes (fluorescence in situ hybridization [FISH]) has enhanced the study of chromosome morphology (Chapter 42).[4] Fluorescently labeled probes allow both specific identification of chromosomes involved in structural or numerical aberrations and analysis of interphase cells. The labeled probe is hybridized directly to cells mounted on glass slides. This procedure preserves the morphologic information of the cell with the mutation. For hybridization to occur, however, the cells must be permeabilized and the DNA denatured. The molecular probe is allowed to hybridize to the chromosomes in the tissue specimen on the slide. Probe binding can be visualized using a fluorescent microscope.

Chromosome Analysis

Conventional cytogenetic analysis and fluorescence in situ hybridization is performed microscopically. The adequacy of conventional cytogenetic analysis depends on the mitotic rate of the cells and on

★ **TABLE 41-2** Cytogenetic Banding Techniques and Special Procedures

Type of Banding or Special Procedure	Procedure Description	Result
Q banding	Quinacrine fluorescence A-T rich areas = bright G-C rich areas = dull	Reveals distinct bright and dull band patterns of homologous chromosomes
G banding	Giemsa stain after enzyme pretreatment A-T rich areas = dark G-C rich areas = pale	Reveals distinct dark and pale band patterns of homologous chromosomes identical to Q bands
R banding	A-T rich areas = pale G-C rich areas = dark	Reveals distinct pale and dark band patterns of homologous chromosomes reverse of Q and G bands
C banding	Giemsa stain after acid-alkali denaturation	Stains heterochromatic pericentric regions of chromosomes 1, 9, 16, and long arm of Y chromosome
NOR staining	Silver stain of nucleolar organizing region	Stains stalk region of acrocentric chromosomes 13, 14, 15, 21, and 22
FISH	Fluorescently labeled DNA probes	Depending on probe used, hybridizes with a specific chromosome pericentromeric region, arm, whole chromosome, or gene
Synchronization	Synchronization of cells in cell cycle with blocking agent (high-dose thymidine)	Increases number of cells in mitosis
High-resolution banding	Cells synchronized and stopped in late prophase or early metaphase	Reveals chromosomes that are less condensed, more toward the prometaphase, and banded at a >800 band stage
Fragile site	Cells cultured with folate/thymidine deprivation	Reveals areas of chromosome gaps, previously used for fra(X)
SCE	Sister chromatid exchange detected by incubation through two cell cycles in BrdU and stained with fluorescent; patient and control DNA are amplified and labeled with different fluorophores and hybridized to an array with DNA probes specific for the entire genome or part of the genome (chromosome specific)	Stains sister chromatids dark or light showing areas of exchange, indicating mutagenicity
CMA		Differential in signal intensity (patient to control) is indicative of copy number gain or loss

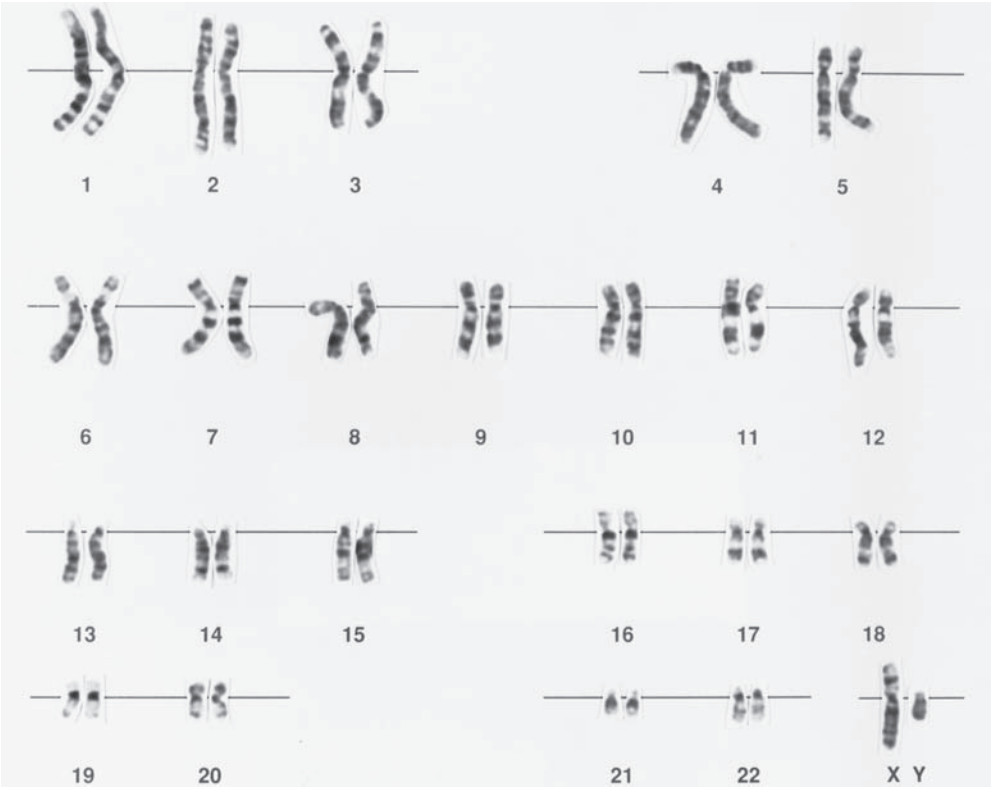
FISH = fluorescence in situ hybridization; NOR = nuclear organizing region; SCE = sister chromatid exchange; CMA = cytogenomic microarray analysis

banded chromosome morphology. An optimal preparation has mitotic spreads with moderately long chromosomes, few chromosome overlaps, and good quality banding (Figure 41-5 ■). A **karyotype** is a representation of the chromosome makeup of a cell that is constructed using a video-computer-linked analysis system. To prepare a karyotype, the chromosomes are grouped initially by size and centromere position and then by the specific pattern of dark-to-light–staining bands (Figure 41-6 ■). The number of cells analyzed per case varies according to the clinical situation. Accrediting agencies such as the College of American Pathologists (CAP) have set standardized guidelines for cytogenetic evaluation.

☑ CHECKPOINT 41-1

A newborn baby boy has multiple congenital malformations, and a chromosome abnormality is suspected as the cause. What is the most appropriate specimen to submit for chromosome analysis, and how should the laboratory professional process the specimen?

■ **FIGURE 41-5** Metaphase spread of chromosomes belonging to a single cell obtained by direct harvest of a bone marrow aspirate (G-banded, 1000× magnification).



■ **FIGURE 41-6** Karyotype of normal male cell with numbered chromosome pairs, G-banded.

CHROMOSOME ABNORMALITIES

Chromosome abnormalities are either numerical or structural and can involve the autosomes (1–22) and/or the X and Y sex chromosomes. Constitutional abnormalities are present at the time of birth and in all cells if they are inherited from a parent carrier or if they occurred during gametogenesis. Constitutional aberrations can also occur in the embryo shortly after fertilization resulting in a **mosaic**: Some cells have the aberration and some are normal. If the aberrations occur some time after birth, they are acquired, as is usually seen in a single cell line identifying a neoplastic clone.

Numerical Aberrations

A normal human cell with 46 chromosomes is called **diploid**. The word **haploid** designates half the number of chromosomes, 23, and *n* is an abbreviation for the haploid number. Therefore, a cell with 2n (2 × 23) has 46 chromosomes (diploid), and a cell with 3n has 69 chromosomes (triploid). **Aneuploid** refers to a chromosome count other than 46 that is not a multiple of n. If a cell has more than 46 chromosomes, the word **hyperdiploid** is used, and if a cell has fewer than 46 chromosomes, it is called **hypodiploid**.

It is thought that a process of **nondisjunction** causes most numerical aberrations. It occurs during meiotic or mitotic cell division when a spindle fiber from the centriole does not connect to the chromosome centromere or when the spindle fiber connects but does not contract (Figure 41-7 ■). This situation results in one daughter cell with an extra chromosome (**trisomy**) and one daughter cell with a chromosome loss (**monosomy**). In most cases, the cell with the chromosome loss does not survive the next cell cycle. Another process, termed *anaphase lag*, results when one chromatid does not completely migrate to the opposite pole but lags behind and gets caught outside the nuclear membrane (Figure 41-8 ■), yielding one daughter cell with a chromosome loss and one daughter cell that is normal.

The word **polyploid** refers to cells that have a chromosome count that is a multiple of the n. Hence, a *tetraploid* cell is polyploid and has a chromosome count of 4n, or 92. **Endomitosis** is the process that results in polyploid cells when there are multiple rounds of S phase (DNA synthesis) without karyokinesis (nuclear division) or cytokinesis (cytoplasmic division). The megakaryocyte is an example of a normal polyploid cell. The word **pseudodiploid** is used when a cell has a chromosome count of 46 but is not normal because of numerical and/or structural aberrations. For example, a cell with a karyotype of 46,XX,+8,−21 is an abnormal cell but has a chromosome count of 46 and therefore is pseudodiploid: it has 46 chromosomes including the 2 sex chromosomes, XX, but it is missing a number 21 chromosome (−21), and has 3 number 8 chromosomes (+8).

Structural Aberrations

Structural chromosome aberrations result when chromosome breakage occurs and the repair process results in structural loss or in abnormal recombinations. Table 41-3 ★ lists structural aberrations with a short description and example nomenclature. Any of these can be seen as constitutional or acquired with the exception of homogeneously staining regions and double minutes that have been seen only in neoplastic cells as acquired aberrations.

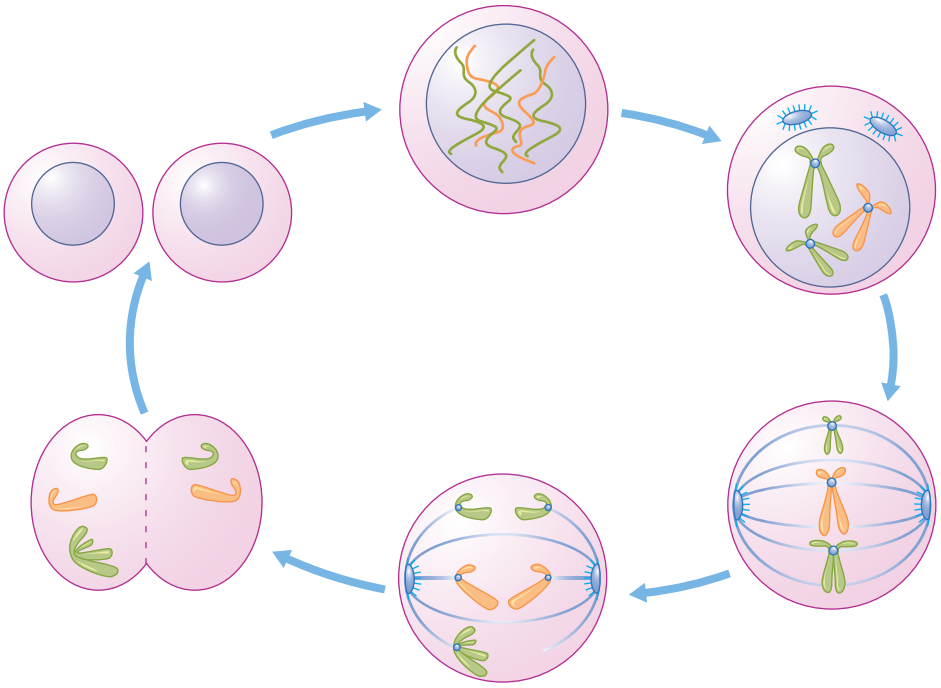


FIGURE 41-7 Nondisjunction. During anaphase, the sister chromatids of a chromosome do not disjoin, resulting in one daughter cell with an extra chromosome (trisomy) and the other with a chromosome loss (monosomy).

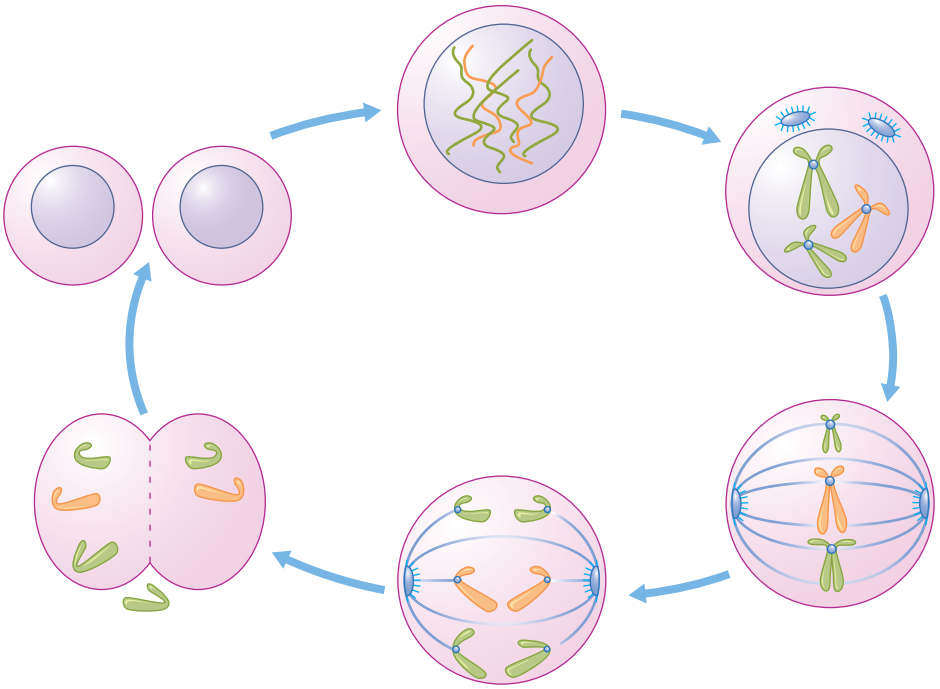


FIGURE 41-8 Anaphase lag. Chromatid does not complete migration, resulting in one daughter cell with a normal chromosome count and the other with a chromosome loss.

★ **TABLE 41-3** Examples of Structural Chromosome Aberration Nomenclature with Explanations

Structural Aberration and Nomenclature	Explanation
Chromosome/chromatid breaks	Break occurs in chromosome/chromatid and is usually repaired. Increased random breaks can be seen with toxins, radiation, and virus exposure.
Dicentric (dic) example: dic(7;8)(q32;q23)	Breaks occur in two chromosomes and the chromosomes—including centromeres—are repaired together resulting in a chromosome with two centromeres; the acentric (lack of a centromere) fragments are lost.
Double minutes (dmin)	Small acentric pieces of DNA, usually paired, indicate gene amplification.
Homogeneously staining region (hsr) example: hsr(11)(q23)	Region of chromosome that stains homogeneously and indicates gene amplification.
Deletion (del), interstitial example: del(7)(q31q32)	Two breaks occur in one arm and material between breaks is lost; break ends are repaired by joining together.
Deletion, terminal example: del(7)(q32)	Break occurs and acentric fragment is lost during mitosis/meiosis.
Duplication (dup) example: dup(7)(q31q32)	Region of a chromosome is duplicated and can be direct or inverted.
Isochromosome (i) example: i(7)(p10) or i(7)(q10)	Centromere splits horizontally and results in chromosome with only short or long arm material. The remaining arm of the chromosome is usually lost.
Inversion (inv), paracentric example: inv(7)(q21q32)	Two breaks occur, and the material between the breaks inverts and then is repaired. When the inversion does not involve the centromere, it is referred to as a paracentric inversion.
Inversion, pericentric example: inv(7)(p15q21)	Two breaks occur, and the material between the breaks inverts and then is repaired. When the inversion involves the centromere, the resulting chromosomal aberration is referred to as a pericentric inversion.
Ring chromosome (r) example: r(7)(p21q35)	Breaks occur in the short and long arms, and the broken ends are repaired together; the acentric fragments are lost.
Translocation (t), balanced example: t(7;8)(q32;q23)	Breaks occur in two different chromosomes with fragments repaired (joined) to the opposite chromosome; no loss of DNA occurs.
Derivative (der) chromosome example: der(7)t(7;8)(q32;q23)	This is a structurally rearranged chromosome derived most often from two or more chromosomes. In this example, the derivative, der (7), is the abnormal chromosome 7 that results from a translocation between chromosome 7 and 8 at the designated break points.
Translocation, Robertsonian example: der(14;21)(q10;q10)	A unique type of translocation, breaks occur at or near the centromeres of two acrocentric chromosomes; the centromeric regions fuse, and the short arm/satellite material is lost. The chromosome is derived (der) from the long arms of each chromosome.

Polymorphic Variation

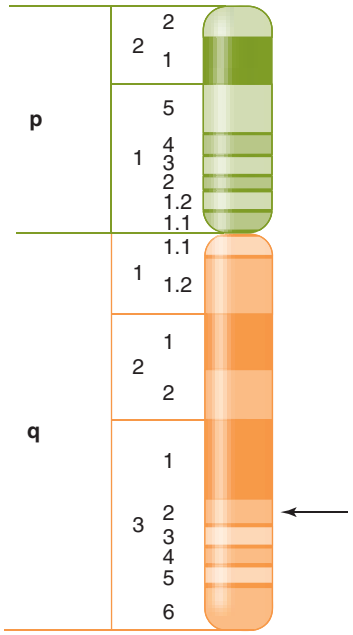
Morphologic variations are known to occur in certain chromosomes. These variations have no clinical significance but, if present, will be inherited consistently through each generation. **Polymorphic variants** (chromosomes with variant morphology that have no clinical consequence) are easily demonstrated with various banding techniques and can be used to identify maternal versus paternal origin of homologous chromosomes. Some of the more common polymorphic variants include a pericentric inversion of chromosome 9; variable amounts of pericentric heterochromatin on chromosomes 1, 9, and 16; and a variable amount of heterochromatin on the long arm of the Y chromosome. Also, amounts of satellite material on the short arms of the acrocentric chromosomes can be variable.

CYTOGENETIC NOMENCLATURE

An international committee promoting one standardized nomenclature system has established the designation of chromosome number, region, band, and karyotype nomenclature. The International System for Human Cytogenetic Nomenclature (ISCN) has published guidelines including specific rules for cancer cytogenetics for use by clinical and research laboratories.[5] The short and long arms of each chromosome are divided into regions by major landmark bands (Figure 41-9 ■). Each region is further divided into distinct light-, intermediate-, and dark-staining bands. The numbering of regions and bands begins at the centromere and proceeds distally to the terminal portions, *pter* and *qter*. The numbering of bands begins with the number 1 for each region. To designate a specific band of a chromosome, the order is written as chromosome number, arm, region, and band.

A standardized nomenclature system also exists for gene names. The HUGO Gene Nomenclature Committee (HGNC) is the recognized body that approves a gene name and symbol for each known human gene. All approved symbols are stored in the HGNC database. Approved gene names and symbols are readily accessible electronically.[6]

The karyotype of a cell is designated first by the total number of chromosomes followed by the sex chromosomes (XX for female and XY for male). If aberrations are present, sex chromosome abnormalities are listed first followed by abnormalities of autosomes listed in numerical order of the chromosomes involved. Numerical abnormalities are designated by + or − before the chromosome number. Structural abnormalities are listed by the appropriate abbreviation followed by the chromosomes involved in parenthesis and then by the break point band designation in parentheses. Therefore, normal female and normal male karyotypes, respectively, are 46,XX and 46,XY. A male cell with trisomy for chromosomes 8 and 21 is designated: 48,XY, +8, +21. A female cell with trisomy for chromosomes 3, 8, and 15 and a translocation involving chromosomes 9 and 22 is designated 49,XX,+3,+8,t(9;22)(q34;q11.2),+15. The q34;q11.2 refers to the long arms (q), regions 3 and 1, and bands 4 and 1.2, respectively. (See Table 41-3 for other examples of nomenclature.)



■ **FIGURE 41-9** Diagram of bands on chromosome 7 with arm, region, and band designations. The band located at the arrow is designated 7q32.
Short arm = p; long arm = q

CYTOGENETIC ANALYSIS OF HEMATOPOIETIC AND LYMPHOID DISORDERS

Cytogenetic analysis has become an essential part of the diagnostic evaluation of patients with known or suspected neoplasms. Many chromosome aberrations ascertained by either conventional (G-banded) techniques or FISH are now considered diagnostic of or have significant prognostic implications for hematolymphoid malignancies[7] and solid tumors.[8] The cloning of genes at critical chromosome breakpoints in various neoplasms has permitted the discovery of the role that these genes play in tumorigenesis and has provided specific DNA sequence targets that can be used for the molecular cytogenetic technique of FISH for patient diagnostics. A chromosome aberration found in neoplastic cells is referred to as an *acquired, clonal aberration*; it is an aberration that occurs sometime after birth and is present only in the neoplastic cells.

A clone exists if numerical and/or structural aberrations are identical in at least two cells unless the abnormality is a single chromosome loss (monosomy); then three cells must have the same chromosome loss. The presence of an abnormal clone is evidence of a neoplasm. Table 41-4 ★ lists the most common uses of cytogenetic analysis in hematolymphoid disorders.

★ **TABLE 41-4** Present Applications of Conventional Cytogenetics and Molecular Cytogenetics (FISH) in Hematolymphoid Disorders

- Confirm or establish the diagnosis of a number of leukemias and lymphomas
- Predict response to specific agents such as tyrosine kinase inhibitors
- Confirm or predict accelerated phase of CML
- Confirm or establish remission and monitor minimal residual disease
- Aid in the diagnosis and prognosis of myelodysplastic states
- Evaluate bone marrow transplant for donor versus recipient cells and possible recurrence of original neoplasm.
- Evaluate clonal evolution, which portends a more aggressive phase of disease.

☑ CHECKPOINT 41-2

Cytogenetic studies were performed on a bone marrow specimen from a female patient with a myelodysplastic state with the following results:

1 cell — 47,XX,+8

1 cell — 45,XX,−20

5 cells — 46,XX,del(5)(q13q34),−7,+21

Which of these aberrations is clonal? Why? What term would apply to the five cells?

Constitutional chromosome aberrations occasionally are found when analyzing neoplastic cells. Most often these are constitutional abnormalities that are not associated with an abnormal phenotype. For example, a female patient with acute leukemia and a 47,XXX karyotype could have the +X as an acquired aberration indicative of the malignant cells, or the +X could represent a constitutional abnormality and be present in all of the patient's cells. If the karyotype is 45,XX,−7, however, this is most likely an acquired aberration because monosomy 7 as a constitutional aberration is not compatible with life. The presence of constitutional aberrations must be accurately interpreted and distinguished from acquired clonal aberrations. This is most often accomplished by stimulating peripheral lymphocyte analysis or occasionally by performing skin biopsy fibroblast culture because these cells are not part of the neoplastic clone and aberrations that are present are constitutional.

Processing Specimens

The best sample for cytogenetic analysis of hematolymphoid disorders, excluding lymphomas, is a bone marrow aspirate; even when blast cells are present in the peripheral blood, a higher mitotic rate is usually achieved from the bone marrow sample. These cells are processed by direct harvest and/or unstimulated cultures. The overall cellularity of the marrow aspirate or peripheral blood sample can vary greatly and affects the mitotic yield and chromosome morphology. Therefore, it

is best for the cytogenetic laboratory to evaluate the cell count of each specimen, usually with an automated cell counter. Optimal cultures are obtained by inoculating 1 million cells per milliliter of media. The best specimen for study of lymphoma is an involved lymph node. In general, the resolution of chromosomes from an unstimulated peripheral blood or bone marrow/lymph node specimen is not as good as that from a phytohemagglutinin-stimulated study, which can reflect an inherent feature of the malignant cells. In fact, cells with particularly poor morphology can represent the abnormal clone, whereas the cells with better morphology can be the remaining normal population. When working in cancer cytogenetics, therefore, the laboratory professional must be careful to analyze each mitotic spread and to evaluate the metaphase preparations with poor resolution and morphology.

Processing cells from lymphoma can include incubation with mitogens in addition to the unstimulated cultures. The neoplastic cells from mature B-cell lymphomas often respond to pokeweed or lipopolysaccharide antigens and to stimulation with phytohemagglutinnin in the presence of interleukin-2. Table 41-5 ★ provides a summary of specimen processing.

Chronic Myelogenous Leukemia

The first chromosome abnormality reported to be associated with a malignancy was described in 1960 as an abnormally small chromosome seen in patients with chronic myelogenous leukemia (CML).[9] This abnormality was designated the Philadelphia or Ph[1] (now designated as Ph) chromosome and was believed to be the result of a deletion of the long arm of chromosome 22. With the advent of banding techniques, the abnormality was found actually to be a balanced translocation involving chromosomes 9 and 22, t(9;22)(q34.1;q11.2).[10] This translocation is seen in approximately 90–95% of patients with CML.

Investigators attempted for some time to decipher why a specific chromosome translocation should be so closely associated with a single morphologic type of leukemia. It is now known that the proto-oncogene *ABL1*, normally located at 9q34.1, is translocated and juxtaposed next to the *BCR* gene at 22q11.2 in the Philadelphia translocation.[11] A proto-oncogene is a normal gene involved in cell division/proliferation that has the capability of becoming an oncogene (Chapters 23, 42). The Ph translocation results in a new chimeric gene consisting of a portion of the *ABL1* from chromosome 9 and a portion of the *BCR* from chromosome 22. The *ABL1* is activated to a functioning oncogene, and a 210 kD (kD = kiloDaltons) polypeptide product of *BCR/ABL1* is present in the leukemic cells.[12] An oncogene is involved in deregulating cell growth, proliferation, and differentiation and is responsible for neoplastic proliferation usually by mutation, overexpression, or amplification.

Of CML patients, 5–10% lack the classic t(9;22) and instead have a variant translocation involving at least one other chromosome in addition to chromosomes 9 and 22 or a cryptic translocation of chromosomes 9 and 22. *Cryptic translocations* are molecular genetic rearrangements that cannot be identified by conventional cytogenetics and must be evaluated by a molecular technique such as FISH (Figure 41-10 ■) or the reverse transcriptase polymerase chain reaction (rtPCR) for *BCR/ABL1* gene rearrangement. Patients with variant or cryptic translocations have the same clinical features and prognosis as those with the typical (9;22) translocation (Chapter 42).

In the past, CML was difficult to treat, and patient survival was poor because the t(9;22) and concurrent *BCR/ABL1* gene rearrangement occur in a pluripotent hematopoietic stem cell (HSC) and has the potential to affect all marrow cells derived from that HSC. The identification of the *BCR/ABL1* fusion transcript and the subsequent cloning of these genes permitted an understanding of the endogenous tyrosine kinase activity of *BCR/ABL1* and prompted the development of an agent targeted specifically at the protein product of the *BCR/ABL1* gene (Chapter 24). This group of agents, called *tyrosine kinase inhibitors (TKIs)*, is now a model for the development of novel treatments in various types of cancer because genetic rearrangements of many other hematolymphoid malignancies and solid tumors are known to involve genes also with tyrosine kinase activity. The TKIs have been shown to significantly prolong overall survival in a number of leukemias, lymphomas, and solid tumors and have revolutionized the treatment of these neoplasms by targeting specific genetic loci.[13,14]

When CML patients enter the accelerated phase or blast crisis, the majority have a change in the karyotype with chromosome aberrations in addition to the t(9;22) (i.e., clonal evolution). The most frequently

★ **TABLE 41-5** Appropriate Specimens and Type of Processing for Cytogenetic Analysis of Neoplastic Cells

Possible Diagnosis	Specimen	Processing
Acute leukemias (myeloid and lymphoid)	Bone marrow at diagnosis	24–72-hour unstimulated cultures; FISH analysis with appropriate probes
Myeloproliferative neoplasms Myelodysplastic syndromes Therapy-related myeloid disorders	Bone marrow at diagnosis; peripheral blood for FISH evaluation to monitor minimal residual disease	Unstimulated cultures
Lymphoproliferative neoplasms	Morphologically involved lymph node, spleen, bone marrow, peripheral blood	Unstimulated cultures; T cell: phytohemagglutinin stimulation; B cell: pokeweed, lipopolysaccharide, PHA, and IL-2
Solid tumor	Morphologically involved tumor tissue	Monolayer and/or suspension cultures
	Effusion (pleural, peritoneal, etc.)	Direct harvest, unstimulated cultures

FISH = fluorescence in situ hybridization; PHA = phytohemagglutinin; IL-2 = interleukin-2

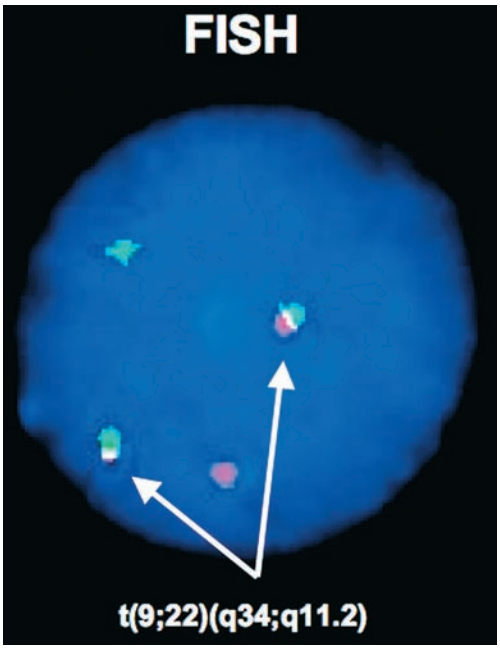


FIGURE 41-10 Identification of the *BCR/ABL1* gene rearrangement with a dual color, dual fusion probe set. The *ABL1* probe is labeled in red and the *BCR* probe in green. One normal *ABL1* gene and one normal *BCR* gene are located on separate chromosomes. The *BCR* and *ABL1* genes are juxtaposed on the two chromosomes involved in the translocation, resulting in two yellow fusion signals.

observed additional aberrations are an extra Ph [+der(22)t(9;22)], an extra chromosome 8 [+8], an isochromosome for the long arm of chromosome 17 [i(17)(q10)], and an extra chromosome 19 [+19]. This clonal evolution can be detected days or weeks before the actual morphologic transformation to blast crisis is seen in the marrow or peripheral blood, allowing more rapid institution of appropriate treatment.[15]

The t(9;22) is also seen at diagnosis in approximately 15% of cases of acute lymphoblastic leukemia (ALL), more commonly in adults (Chapter 24). Although the translocation appears the same cytogenetically as that seen in CML, there is a difference in the site of breakage at the *BCR* locus, resulting in a 190 kD protein. The cases of Ph-positive ALL must be distinguished from a patient presenting in lymphoid blast crisis of CML because the prognosis and treatment are different.

CASE STUDY *(continued from page 873)*

Cytogenetic analysis of bone marrow shows all of the cells to have this karyotype: 46,XY,t(9;22)(q34.1;q11.2).

2. Is this a constitutional or acquired aberration?
3. Is it a clonal aberration?
4. What is the significance of this finding for diagnosis?

Myeloproliferative Neoplasms Other Than CML

The other myeloproliferative neoplasms, polycythemia vera (PV), chronic idiopathic myelofibrosis, and essential thrombocythemia (ET), have acquired clonal chromosome aberrations in approximately 50–60% of cases. Diagnosis of these disorders now requires documented absence of *BCR/ABL1* gene rearrangement.[7] Recurrent aberrations in these myeloproliferative neoplasms include abnormalities of 1q, +8, +9, del(13q), and del(20q). In addition to negative *BCR/ABL1* gene rearrangement status, molecular genetic evaluation for a particular mutation in the *JAK2* gene, the V617F mutation, has become the standard of care for the diagnosis of these patients (Chapter 24). Patients who have the V617F mutation can definitively be diagnosed with a myeloproliferative neoplasm, have a high response rate to a particular chemotherapeutic agent called *hydoxyurea*, and have been shown to have shorter overall survivals.[16]

Acute Myeloid Leukemia

Approximately 70% of patients with de novo acute myeloid leukemia (AML) have acquired clonal chromosome aberrations in the leukemic cells. These aberrations are present only in the leukemic blasts, not in the other hematopoietic precursors. Therefore, de novo AML appears to be a malignancy of the committed progenitor cell.[17,18] The chromosome abnormalities found can be single, numerical, or structural or complex. Some aberrations such as trisomy 8 occur frequently in AML but are not diagnostic for a specific type of leukemia. When present in the diagnostic specimen, chromosome aberrations can be valuable in following the leukemia's progression or regression. If the original leukemic cells have a clonal aberration, such as +8, a complete remission sample should have only a normal karyotype. In subsequent samples, the presence of even one cell with +8 would indicate an early relapse, which might not be detectable morphologically. Some patients clonally evolve, usually indicating cytogenetic transformation to a more aggressive and treatment-resistant disease.

The World Health Organization (WHO) has established a classification system for AML according to the presence or absence of recurrent cytogenetic abnormalities[7] (Chapters 23, 26). Specific cytogenetic aberrations now define particular subtypes of AML that have specific treatment implications. Although particular morphologic and clinical features characterize these cytogenetic entities, the primary diagnostic modality is genetic. The WHO AML classification includes the aberrations in Appendix C. If a patient with AML lacks one of these recurrent aberrations or other clonal cytogenetic abnormality, classification is based on morphologic and cytochemical/immunophenotypic features (Chapter 26).

The t(8;21) and inv(16)/t(16;16) both disrupt the same transcription factor, core binding factor (CBF). The t(8;21) disrupts the α-chain of CBF that is encoded by *RUNX1*, and the inv(16)/t(16;16) disrupts the β-chain of CBF. These leukemias have relatively favorable prognoses, are more common in younger patients, and can have a unique type of presentation with a particular type of disease called *myeloid sarcoma*, a soft tissue infiltration by the myeloblasts.

The t(8;21), previously reported predominantly in cases of AML-M2 by French–American–British (FAB) classification (AML

with differentiation) occurs in 5–12% of cases of AML. The protein product of the *RUNX1T1/RUNX1* gene rearrangement promotes leukemogenesis by blocking myeloid cell differentiation.[19] A loss of a sex chromosome, Y in the male and X in the female, frequently seen with the t(8;21). FAB classification previously reported the inv(16)/t(16;16) in cases of AML-M4eo (acute myelomonocytic leukemia with increased marrow eosinophils), which occurs in 10–12% of AML.[7,20,21] The t(15;17) is present in acute promyelocytic leukemia (APL), previously classified as AML-M3 by FAB, and accounts for 5–8% of AML. The translocation results in the fusion of the retinoic acid receptor alpha (*RARA*) gene at 17q21.1 with the nuclear regulatory factor promyelocytic leukemia (*PML*) gene at 15q24.1. These patients respond to treatment with all transretinoic acid (ATRA), which induces differentiation of the abnormal promyelocytes. This treatment is followed by chemotherapy with an anthracyclin agent. Four variant aberrations also rearrange the *RARA* gene but involve different partner chromosomes (Appendix B). Identification of these variant translocations is clinically important because some resist treatment with ATRA, whereas others respond.[7]

AML with the t(8;21), inv(16) or t(16;16), and t(15;17) are considered acute leukemias without regard to blast count. It is still unclear whether cases with the t(9;11), t(6;9), inv(3) or t(3;3), and t(1;22) should be considered AML when the blast count is less than 20%. The WHO also recognizes an entity of AML with myelodysplasia (MDS)-related changes that is characterized by a blast count of >20% in the blood or bone marrow, morphologic features of MDS or a history of MDS, MDS-related cytogenetic abnormalities (often complex including chromosome 5 and 7 aberrations), and an absence of the aberrations seen in AML with recurrent genetic abnormalities. These patients have a poor prognosis with decreased likelihood of achieving complete remission and short overall survival. Therapy-related myeloid neoplasms occur in patients who have previously received chemotherapy or radiation therapy. These cases are called *secondary* or *therapy-related myeloid disorders* and are considered as a distinct entity by the WHO. Although in the 2001 WHO classification system the therapy-related AMLs were subcategorized as "alkylating agent related" or topoisomerase II-related or "other," in the 2008 classification system, they are no longer subcategorized. However, the specific genetic abnormality found in these disorders should be listed. Alkylating agent-related myeloid disorders occur approximately 5–6 years after treatment and are associated with aberrations of chromosomes 5 and 7. Topoisomerase II inhibitor-related myeloid disorders present within 2–3 years of exposure and are associated with break points at band 11q23 and concurrent *MLL* gene rearrangement.[7,22]

☑ CHECKPOINT 41-3

A 35-year-old man has acute leukemia. Cytogenetic studies of the bone marrow reveal the following: 46,XY,t(15;17)(q24.1;q21.1). What type of leukemia does this patient have, and what will cytogenetic studies show after treatment if he achieves complete remission?

Myelodysplastic Syndromes (MDSs)

MDSs are clonal neoplastic disorders of hematolymphoid stem cells characterized by dysplasia and subsequent ineffective hematopoiesis in two or more myeloid cell lines (Chapter 25). An internationally recognized scoring system for predicting survival of MDS patients and transformation to AML, the International Prognostic Scoring System (IPSS), has been developed; its major prognostic variables are the type of cytogenetic abnormality, percentage of blasts, and degree and number of cytopenias. According to the scoring system, good-risk cytogenetics include a normal karyotype, del(5q) as the sole abnormality, del(20q) as the sole abnormality, and −Y. Poor-risk cytogenetics include three or more chromosome aberrations and chromosome 7 abnormalities. Intermediate risk refers to all other abnormalities. These specific cytogenetic aberrations define particular categories of MDS and play a major role in diagnosis, treatment, and management of patients with them.[23]

☑ CHECKPOINT 41-4

Five years ago, a 46-year-old woman received chemotherapy and radiation treatment for breast cancer. She now has pancytopenia. Cytogenetic analysis of the bone marrow is performed and shows 10 of 20 cells with the following: 45,XX,del(5)(q13q34),−7. What is the significance of this finding?

Acute Lymphoblastic Leukemia (ALL)/Lymphoma

Approximately 60–75% of patients with ALL have clonal acquired aberrations of the malignant cells. Cytogenetic findings are a crucial part of the leukemia workup for pediatric cases, and the results are directly related to prognosis. Patients who have the most favorable prognosis have hyperdiploid karyotypes with a chromosome count ranging from 54–65 with concurrent trisomies of chromosomes 4, 10, and 17. Most cases of hyperdiploid ALL have other clinically favorable findings such as 3–7 years of age, total leukocyte count less than 10×10^9/L, and precursor B-cell immunophenotype with CD10 positivity. The t(12;21)(p13;q22.3) also confers a favorable prognosis. It rearranges *ETV6* at 12p13 and *RUNX1* at 21q22.3, and it is a cryptic aberration, undetectable in conventional metaphase preparations because the size of the translocated segments are similar in size and staining intensity. To ascertain the presence of this rearrangement, molecular methods, either FISH or rtPCR, must be employed. Hypodiploidy, the t(4;11)(q21;q23) that rearranges the *AF4* and *MLL* genes and the t(9;22)(q34.1;q11.2) that rearranges the *BCR* and *ABL1* genes are associated with a poor outcome.[7,24,25]

The patient's age influences the prognostic impact of these cytogenetic aberrations. ALL patients with the t(9;22) ranging in age from 1–18 years have more favorable outcomes than adults. Infants and adults with the t(4;11) have significantly shorter overall survivals than children with the same translocation. See Appendix C for some of the more commonly seen aberrations in ALL.

☑ CHECKPOINT 41-5

A 5-year-old girl has fatigue and easy bruising. A CBC shows a leukocyte count of 40 × 10^9/L with 85% blasts. Bone marrow cytogenetic studies are performed and show all cells with the following karyotype: 53,XX, +X,+4,+6,+10,+18,+20,+21. What is the prognostic significance of this finding?

Lymphoma and Lymphoproliferative Disorders

In recent years, chromosome analysis in lymphoma and lymphoproliferative disorders has added greatly to the understanding of the importance of the gene loci involved in chromosome aberrations seen with specific types of lymphoma. The results of these analyses have also led to the development of molecular probes and their use in the clinical laboratory. The first abnormality described in the lymphomas was the 14q+ seen in Burkitt lymphoma.[26] This was later characterized as t(8;14)(q24.1;q32.3) and is seen in approximately 75% of cases of Burkitt lymphoma. Two variant translocations, t(2;8)(p11.2;q24.1) and t(8;22)(q24.1;q11.2), have been reported in 10–15% of Burkitt lymphoma. The 14q32.3 is the site of the gene for the heavy chain of immunoglobulin, and the breakpoint at 8q24.1 is just proximal to the site of the proto-oncogene *MYC*. Therefore, the *MYC* gene is translocated to the heavy chain locus at 14q32.3.[27] This was the first demonstration of a proto-oncogene that was translocated to a location known to be active in B-cell lymphoma, resulting in the activation of the oncogene. The t(2;8) results in the juxtaposition of a gene for κ light chain to *MYC*, and the t(8;22) results in the juxtaposition of a gene for λ light chain to *MYC*. In cases of Burkitt lymphoma that have the two variant translocations, the tumor cells are found to mark with surface κ-chain when the t(2;8) is found and with λ-chain when the t(8;22) is found.

Many cytogenetic aberrations are now associated with specific types of lymphoma and play a major role in diagnosing these entities as well as having prognostic significance. Most of these translocations are known to involve genes that are critical for proliferation of the neoplastic cells or are involved in programmed cell death, apoptosis. Chronic lymphocytic leukemia (CLL) patients with trisomy 12, 11q, or 17p aberrations are associated with an atypical morphology and shorter overall survival.[28] The t(11;14) is diagnostic of mantle cell lymphoma, an aggressive entity that requires chemotherapy and transplantation, in the appropriate morphologic and clinical context.[29] The presence of the t(11;18) or t(1;14) in extranodal gastric lymphoma of mucosa-associated lymphoid tissue (MALT), a type of marginal zone lymphoma, defines it as malignancy and requires treatment with chemotherapy as opposed to antibiotics.[30] Patients with anaplastic large cell lymphoma bearing the t(2;5) have a significantly longer survival than those patients without this aberration.[31] See Appendix C for the characteristic chromosome aberrations with the lymphoproliferative disorders and gene loci known to be involved in these rearrangements.

BONE MARROW TRANSPLANTATION

Some of the hematolymphoid disorders presented in this chapter are treated by bone marrow or peripheral blood stem cell transplantation, depending on the clinical situation and availability of donors. Cytogenetics, particularly the molecular cytogenetic technique of FISH, is a valuable tool in evaluating the rate of engraftment of the donor cells in opposite sex transplants and monitoring for minimal residual disease. Following successful transplantation, only donor cells should be present. Occasionally, 1–2% of recipient cells are detectable shortly after transplant but then diminish. Recipient cells that recur with a normal karyotype indicate the development of a **chimerism**, the presence of cells of two different genetic origins in an individual. For example, a female recipient after a male-donated transplant can show 30% XY and 70% XX by FISH analysis with probes for the X and Y centromeres, which is consistent with partial but not complete engraftment. Treatment to promote engraftment, such as increasing the immunosuppression or giving additional donor cells, can thus be employed. If the recipient marrow cells have an initial chromosome aberration, FISH of DNA probes specific for the genes that are rearranged by that aberration can be used to assess the degree of disease burden persisting.

CASE STUDY *(continued from page 880)*

An allogeneic bone marrow transplant is performed with Gregory's sister as the donor. Three months after transplant, the karyotype is 46,XX.

5. What is the significance of this finding?

MOLECULAR CYTOGENETICS

Techniques are now available to evaluate not only the gross chromosome morphology but also the individual gene composition. FISH and cytogenomic microarray analysis (CMA) are examples of such technology. The role FISH plays in the diagnosis and prognosis of patients with hematolymphoid disorders has been previously discussed. The advantage of these techniques over conventional cytogenetic studies is that molecular DNA techniques do not require viable cells capable of mitotic activity. Samples of tumor cells can include nondividing peripheral blood cells and paraffin-embedded tissue. The disadvantage of certain molecular studies, such as FISH, is that they give information about only a single molecular genetic aberration based on the specific probe used, potentially missing other chromosome aberrations that might be present (Chapter 42).

The following scenario illustrates the use of the two different techniques. A 25-year-old man is known to have CML and has received an allogeneic transplant from his sister. The first post-transplant specimen did not have dividing cells in the conventional cytogenetic preparations. FISH of DNA probes that are rearranged in this leukemia, the *BCR* and *ABL1* genes, was done and was positive in 10 of 200 interphase nuclei examined, consistent with a low-level

persistence of disease. A second sample obtained 3 months later was sufficient for conventional cytogenetics and yielded the following results:

80% 46,XX

10% 46,XY,t(9;22)(q34.1;q11.2)

10% 47,XY,+8,t(9;22)(q34.1;q11.2)

In the first sample, the molecular FISH studies were critical to show the persistence of the leukemia. Cytogenetics performed on the second sample was critical to show a cytogenetic transformation toward blast crisis with the additional +8 abnormality. The molecular technique of rtPCR also has significant clinical utility in diagnosing and monitoring patients with hematolymphoid neoplasms including CML (Chapter 42).

Cytogenomic microarray analysis (CMA) is widely used clinically to analyze changes in chromosome copy number (copy number variation). Because of the greatly increased resolution that these assays provide, it has been observed that the human genome has much more variability than previously thought with differences found at up to thousands of loci, most of which are not clinically significant. This variability makes interpretation of CMA potentially challenging. In addition, the microarrays used in CMA have been designed to analyze critical regions of the DNA for well-defined genetic abnormalities. DNA from the patient and normal control DNA are labeled using different fluorescent dyes, mixed, and hybridized to a slide containing hundreds to thousands of defined DNA probes. The color intensity ratio is analyzed along the DNA to detect regions of copy number gain or loss. The current technology is predominantly used for clinical diagnostics of constitutional disorders and detects copy gain and loss of regions that are approximately 10 kb in length.[32] In addition, the evolution of microarrays that use single nucleotide polymorphisms (SNPs) allow the absence of heterozygosity (AOH), the presence of only one allele at a specific chromosomal locus, to be detected. AOH is a frequent finding in cancer specimens and can ultimately have diagnostic utility. Although the presence of complex copy number variation in conjunction with the current lack of clinical correlation for AOH at many cytogenetic loci makes interpretation challenging, CMA will play a role in the future in the routine molecular profiling of neoplasia.[33]

CASE STUDY *(continued from page 882)*

Six months after transplant, Gregory had bone marrow aspirated for analysis. The mitotic yield of the specimen was not sufficient for routine cytogenetic analysis.

6. What other studies that would be informative as to the status of the donor and recipient cells could be performed?
7. Five years after the transplant, cytogenetic analysis shows the following:

   5 cells—46,XX
   15 cells—47,XX,+8,t(9;22)(q34;q11.2),i(17)(q10)

   What is the significance of these findings?

Summary

Clinical specialists in the hematology laboratory are frequently asked when cytogenetic studies are indicated and what specimens are appropriate to submit. For these reasons, it is important for these specialists to have a basic understanding of the specimen requirements, processing, and clinical indications for chromosome analysis. Acquired clonal chromosome aberrations can be seen in most of the hematolymphoid disorders, including acute and chronic leukemias, myelodysplastic states, myeloproliferative disorders, and lymphomas. These results are used for diagnosis and prognosis. In addition, cytogenetics can be used to follow the progression or regression of a malignant clone if the original pretreatment sample revealed a clonal aberration.

The chromosome aberrations found in hematolymphoid disorders are present only in the malignant cells. Many cytogenetic aberrations are now recognized as diagnostic of particular hematolymphoid neoplasms; for example, the t(15;17) is diagnostic of APL. Identification of these specific aberrations with DNA probes has clinical utility for evaluating the patient's response to treatment and degree of residual disease. The identification of these molecular cytogenetic targets has also lead to more effective treatment for many hematolymphoid neoplasms.

Review Questions

Level I

1. Which of the following is the major component of chromosomes? (Objective 1)

   A. nucleotides
   B. enzymes
   C. lipids
   D. proteins

2. In which stage of mitosis is the chromosome morphology best observed? (Objective 1)

   A. anaphase
   B. interphase
   C. metaphase
   D. prophase

3. A 4-year-old boy has mental retardation and developmental delay. Which of the following would be the most appropriate specimen for chromosome analysis? (Objective 2)
   A. bone marrow
   B. skin biopsy
   C. peripheral blood
   D. lymph node
4. A 62-year-old man has acute myeloid leukemia. Which of the following would be the most appropriate specimen for chromosome analysis to determine whether acquired chromosome aberrations exist? (Objective 2)
   A. bone marrow
   B. lymph node
   C. peripheral blood
   D. skin biopsy
5. Which of the following is a basic criterion of cells that would be processed for chromosome analysis? (Objective 2)
   A. mitotic activity
   B. protein production
   C. presence of nucleolus
   D. presence of mitochondria
6. In the harvest procedure, cells are stopped in metaphase by which of the following? (Objective 2)
   A. colchicine/colcemid incubation
   B. incubation with hypotonic solution
   C. fixation with Carnoy's fixative
   D. incubation with phytohemagglutinin
7. The ability to identify individual chromosomes depends on which of the following? (Objective 2)
   A. banding
   B. hypotonic incubation
   C. type of specimen
   D. fixation
8. Which of the following terms is appropriate to describe a human cell that has 47 chromosomes? (Objective 3)
   A. diploid
   B. aneuploid
   C. polyploid
   D. normal
9. Which of the following can result in trisomy? (Objective 3)
   A. anaphase lag
   B. endomitosis
   C. nondisjunction
   D. chromosome breakage
10. Which of the following can result in an abnormal amount of cellular DNA? (Objective 3)
    A. balanced translocation
    B. paracentric inversion
    C. pericentric inversion
    D. isochromosome

Level II

Use this case history for questions 1–3.

Stanley, the patient, is a 35-year-old male who presented with a severe nosebleed. He has been previously healthy; however, in the last three weeks, he has noticed easy bruising, and on the day of admission to the hospital, he had a nosebleed that he could not stop. Initial CBC revealed the following:

Hb	10 g/dL (100 g/L)
Hct	30% (0.30 L/L)
MCV	85 fL
WBC	50×10^3/mcL (50×10^9/L)
Platelet count	20×10^3/mcL (20×10^9/L)

The peripheral smear showed the majority of cells to have a high nuclear:cytoplasmic ratio, immature chromatin, and cytoplasmic hypergranulation with azurophilic granules. Preliminary cytogenetic analysis performed on bone aspirate revealed 75% of cells to have t(15;17)(q24.1;q21.1); 25% of cells have a normal male karyotype.

1. The most likely diagnosis is: (Objective 6)
   A. chronic myeloid leukemia
   B. APL
   C. anaplastic large cell lymphoma
   D. secondary (therapy-related) AML
2. The break point on chromosome 17 involves which gene? (Objective 9)
   A. *ABL1*
   B. *MYC*
   C. *BCR*
   D. *RARA*
3. The identification of the cytogenetic aberration in question 2 in patients has led to their treatment with which of the following? (Objectives 5, 6)
   A. interferon
   B. interleukin
   C. all-trans retinoic acid
   D. vitamin K

Use this case study for questions 4–7.

A 16-year-old girl has fatigue and increased bruising. A CBC shows the following:

Hb	7.0 g/dL (70 g/L)
Hct	21% (0.21 L/L)
WBC	35×10^3/mcL (5×10^9/L)
Platelet count	60×10^3/mcL (60×10^9/L)

Differential, bone marrow aspirate:
50% blasts with monocytoid features
15% dysplastic eosinophils with large basophilic granules
10% myelocytes
10% metamyelocytes
13% red blood cell precursors
2% megakaryocytes

4. Which of the following is most likely to be seen on chromosome analysis of the bone marrow cells? (Objective 6)
   A. t(8;21)
   B. t(15;17)
   C. inv(16)
   D. +8
5. Which of the following genes does the chromosome rearrangement identified in question 4 involve? (Objective 9)
   A. *BCR/ABL1*
   B. *MLL*
   C. *CBFB/MYH11*
   D. *ETV6/RUNX1*
6. What is the other AML with a recurrent cytogenetic aberration that involves the same transcription factor as identified in question 5? (Objective 9)
   A. t(8;21)
   B. inv(16)
   C. t(15;17)
   D. 11q23
7. Which of the following is the most likely diagnosis? (Objective 6)
   A. akylating agent-related AML
   B. core-binding factor leukemia
   C. AML with myelodysplasia-related changes
   D. AML of ambiguous lineage
8. Which of the following genetic abnormalities is associated with a good prognosis when it is found in ALL? (Objective 7)
   A. chromosome count >54
   B. t(9;22)
   C. t(4;11)
   D. chromosome count <45
9. Which of the following is associated with a poor prognosis when found in ALL? (Objective 7)
   A. chromosome count >54
   B. t(12; 21)
   C. normal karyotype
   D. t(9;22)
10. Which of the following is associated with Burkitt lymphoma? (Objective 6)
    A. t(8;21)
    B. t(14;18)
    C. t(12;21)
    D. t(8;14)

Companion Resources

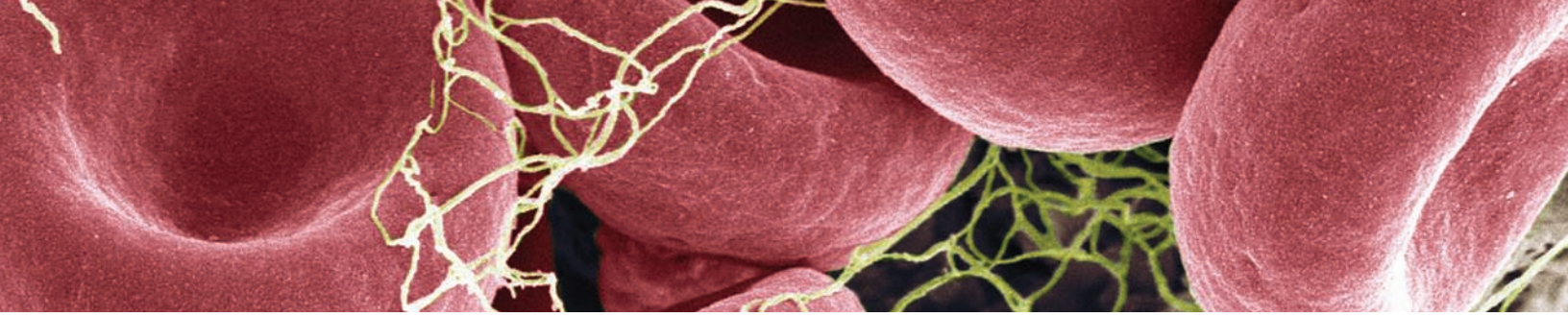
http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Barch M, ed. *The AGT Cytogenetics Laboratory Manual*, 3rd ed. New York: Raven Press; 1997.
2. Caspersson T, Lomakka G, Zach L. The fluorescence patterns of the human metaphase chromosomes-distinguishing characters and variability. *Hereditas*. 1971;67:89–102.
3. Seabright, M. A rapid banding technique for human chromosomes. *Lancet*. 1971;2:971–72.
4. Schad C, Kraker W, Tatal S et al. Use of fluorescent in situ hybridization for marker chromosome identification in congenital and neoplastic disorders. *Am J Clin Path*. 1991;96:203–10.
5. Shaffer LG, Slovak ML, Campbell LJ, eds. *An International System for Human Cytogenetic Nomenclature (2009)*. New York: Karger; 2009.
6. HUGO Gene Nomenclature Committee database. www.genenames.org; accessed March 28, 2012.

7. Swerdlow SH, Campo E, Harris NL et al., eds. *World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th ed. Lyon, France: IARC Press; 2008.
8. Fletcher CDM, Unni KK, Mertens F, eds. *World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Soft Tissue and Bone*. Lyon, France: IARC Press; 2002.
9. Nowell PC, Hungerford DA. A minute chromosome in human, chronic granulocytic leukemia. *Science*. 1960;132:1497.
10. Rowley JD. A new consistent chromosomal abnormality in chronic granulocytic leukemia. *Nature*. 1973;243:290–93.
11. Bartram CR, deKlein A, Groffen J et al. Translocation of C-ABL oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukemia. *Nature*. 1983;306:239–42.
12. Faderl S, Talpaz M, Kantarjian HM et al. The biology of chronic myeloid leukemia. *N Eng J Med*. 1999;341:164–72.
13. Tanaka MF, Kantarjian H, Cortes J et al. Treatment options for chronic myeloid leukemia. *Expert Opin Pharmacother*. 2012;13:815–28.
14. Korpanty G, Smyth E. Anti-VEGF strategies-from antibodies to tyrosine kinase inhibitors: background and clinical development in human cancer. *Curr Pharm Des* 2012;18(19):2680–701.
15. Johansson B, Fioretos T, Mitelman F et al. Cytogenetic and molecular genetic evolution of chronic myeloid leukemia. *Acta Haematol*. 2002;107:76–94.
16. Passamonti F. Prognostic factors and models in polycythemia vera, essential thrombocythemia, and primary myelofibrosis. *Clin Lymphoma Myeloma Leuk*. 2011;11(suppl 1):S25–27.
17. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Eng J Med*. 1999;341:1051–62.
18. Willman C. Acute leukemias: a paradigm for the integration of new technologies in diagnosis and classification. *Mod Pathol*. 1999;12:218–28.
19. Westendorf J, Yamamoto C, Hiebert SW et al. The t(8;21) fusion product, AML-1-ETO, associates with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription, and blocks granulocytic differentiation. *Mol Cell Biol*. 1998;18:322–33.
20. Marcucci G, Mrozek K, Ruppert AS et al. Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): a Cancer and Leukemia Group B Study. *J Clin Oncol*. 2005;23:5705–717.
21. Ravindranath Y, Chang M, Steuber CP et al. Pediatric Oncology Group (POG) studies of acute myeloid leukemia (AML): a review of four consecutive childhood AML trials conducted between 1981 and 2000. *Leukemia*. 2005;19:2101–116.
22. Shali W, Helias C, Fohrer C et al. Cytogenetic studies of a series of 43 consecutive secondary myelodysplastic syndromes/acute myeloid leukemias: conventional cytogenetics, FISH, and multiplex FISH. *Cancer Genet Cytogenet*. 2006;168:133–45.
23. Greenberg P, Cox C, LeBeau MM et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*. 1997;89:2079–88.
24. Pui C-H, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006;354:166–78.
25. Van Grotel M, Meijerkink JPP, Beverloo HB et al. The outcome of molecular-cytogenetic subgroups in pediatric T-cell acute lymphoblastic leukemia: a retrospective study of patients treated according to DCOG or COALL protocols. *Hematologica*. 2006;91:1212–221.
26. Menolov G, Manolaova Y. Marker band in one chromosome 14 from Burkitt lymphoma. *Nature*. 1972;237:33–4.
27. Dalla Favera R, Bregui M, Erickson J et al. Human C-MYC onc gene is located in the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci U S A*. 1982;79:7824–827.
28. Dohner H, Stilgenbauer S, Benner A et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med*. 2000;343:1910–916.
29. Bertoni F, Zucc E, Cavalli F. Mantle cell lymphoma. *Curr Opin Hematol*. 2004;11:411–18.
30. Farinha P, Gascoyne RD. Molecular pathogenesis of mucosa-associated lymphoid tissue lymphoma. *J Clin Oncol*. 2005;23:6370–378.
31. Jaffe ES. Anaplastic large cell lymphoma: the shifting sands of diagnostic hematopathology. *Mod Pathol*. 2001;14:219–28.
32. Miller DT, Adam MP, Aradhya S et al. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet*. 2010;86:749–64.
33. Stricker T, Catenacci DV, Seiwert TY. Molecular profiling of cancer—the future of personalized cancer medicine: a primer on cancer biology and the tools necessary to bring molecular testing to the clinic. *Semin Oncol*. 2011;38:173–85.



42 Molecular Analysis of Hematologic Diseases

SARA TAYLOR, PHD
SALLY LEWIS, PHD

Objectives—Level II

This chapter differs from others in that it has only Level II objectives. The material in this chapter is at the advanced level and requires a background in genetics. At the end of this unit, the student should be able to:

1. Define terms and appropriately use nomenclature associated with molecular pathology.
2. Describe the principles and summarize the procedures for each common laboratory test used in molecular diagnostics of hematopathology.
3. Describe and explain the applications of molecular tests in diagnosing and managing inherited disease, infectious disease, and cancer as they pertain to hematology and hemostasis.
4. Identify common chromosomal translocations and molecular genetic abnormalities associated with chronic myelogenous leukemia, acute leukemia, and lymphoma.
5. Explain how B- and T-cell malignancies can be differentiated from other conditions by molecular testing.
6. Select the most appropriate molecular test for a given patient condition or provisional diagnosis.
7. Compare and contrast the various molecular methods with regard to diagnostic applications and principles.
8. Compare the advantages and disadvantages of molecular tests with other laboratory tests used in diagnosing and managing hematologic disorders.

Chapter Outline

Key Terms

Allele
Complementary DNA (cDNA)
DNA sequencing
Fluorescence in situ hybridization (FISH)
Gene
Gene rearrangement
Genome
Genotype
Hybridization
Mutation
Nucleotide
Polymerase chain reaction (PCR)
Probe
Restriction endonuclease
Southern blot
Transcription
Translocation

Background Basics

This chapter builds on concepts learned in other chapters of this textbook. The reader should have a background of genetic principles. To maximize your learning experience, you should review the following material:

Level II

- Summarize the pathophysiology and etiology of sickle cell anemia. (Chapter 13)
- Describe the role of oncogenes in the etiology and pathophysiology of cancer. (Chapter 23)
- Describe the application of cytogenetic analysis in diagnosing hematologic disease. (Chapter 41)
- Summarize the etiology and pathophysiology of leukemia and lymphoma. (Chapters 23–27)
- Describe the congenital aberrations that lead to hypercoagulability and venous thrombosis. (Chapter 34)

CASE STUDY

We will address this case study throughout the chapter.

Warren, a 59-year-old white male, sees his physician with a complaint of increasing abdominal discomfort. A physical examination reveals that he has an enlarged spleen. His WBC count is 34 × 10^9/L, and the peripheral blood smear shows a left shift and an increased platelet count. His red blood cell (RBC) morphology is described as a normocytic, normochromic cell population. His physician suspects chronic myelogenous leukemia.

Consider the laboratory tests that should be performed to verify the physician's preliminary diagnosis.

OVERVIEW

Molecular diagnostics is essentially the analysis of DNA and RNA at the molecular level for the purposes of elucidating disease etiology. DNA and RNA underlie cell function, and increasingly more pathologies are attributable to genetic mutations. Molecular diagnostics has applications in the identification of infectious agents, patient stratification, drug regimen selection, toxicity avoidance, therapeutic monitoring, and detection of predisposition to disease.

Numerous scientific discoveries needed to take place before the technology that permits molecular diagnosis could develop. Most scientists would agree that Mendel's work elucidating inheritance was among the earliest scientific contributions that lead to our understanding of genetics and that the works of Rosalind Franklin, Maurice Wilkins, James Watson, and Francis Crick lead to an understanding of nucleic acid structure. Nucleic acid sequence and structure are the basis of nearly all molecular techniques (a review of nucleic acid structure and function is provided in brief in Chapter 41). In 1983, a technique, **polymerase chain reaction (PCR)**, that capitalized on nucleic acid structure was developed and revolutionized the way that medicine is practiced. Since then, an explosive increase has occurred in molecular techniques, including an astonishing number of variations of nucleic acid amplification, fluorescent in situ hybridization (FISH), microarray technology, and (next-generation) DNA sequencing. Other advances have been in the areas of miniaturization, automated nucleic acid extraction, and automated analysis—all of which have greatly enhanced the speed, accuracy, and flexibility of molecular diagnostic testing. This chapter describes the most commonly used molecular tests and their application in selected inherited and malignant hematologic and hemostasis disorders.

INTRODUCTION

Undoubtedly, the greatest contribution molecular testing makes to clinical diagnostics is the use of an individual's genetic material for rational decision making about the person's care. Thus, molecular diagnostics is essential in the development of personalized medicine. As its name implies, personalized medicine utilizes each individual's unique clinical, genetic, and environmental information to determine a person's susceptibility to disease, diagnose illness, make treatment decisions, determine response, and to track minimal residual disease. Today patient care is developing along the lines of personalized medicine, a trend that is likely to expand.

At the molecular level, **mutations** are alterations in the portion of DNA molecules that constitute **genes**. DNA is a blueprint of the **genome** and changes to its structure can be of great consequence to the organism. Changes to DNA structure can occur spontaneously (de novo) during DNA replication, recombination, or during repair processes. Mutations can also occur as a result of exposure to chemicals, UV radiation, or ionizing radiation. Recently, epigenetic modifications to DNA have been recognized as contributory to DNA expression (Chapters 2, 23). Epigenetic changes do not involve sequential changes to DNA but silence or allow expression of DNA by well-recognized mechanisms, such as DNA methylation or histone methylation and/or acetylation.

Individuals inherit two **alleles** (one gene from each parent). The most common version of the allele is referred to as the wild type (WT). If a mutation occurs in either allele, the function of the protein

for which it encodes could be altered. The modified function could either be reduced or intensified, so the gene mutation is referred to as a *loss-of-function* or a *gain-of-function mutation*. Loss-of-function mutations might result in impaired biological activity or cancer if the gene (or its encoded protein) lost is a tumor suppressor. Although it is expected that most mutations lead to a loss of function, it is possible that a new and important function could result from the mutation. In these cases, the mutation creates a new allele that produces a protein with new activity, referred to as gain of function. Gain-of-function mutations to genes encoding essential proteins of cell cycling, signaling, and antiapoptosis can lead to dysregulation of these essential cell processes resulting in malignancy (leukemias or lymphomas). When the outcome of gene deviation is cancer, the altered gene is referred to as an *oncogene*; its normal counterpart is known as a *proto-oncogene* (Chapter 23).

Gross chromosome alterations such as deletions, insertions, and **translocations** are demonstrated well with karyotyping. However, subtle mutations such as single nucleotide polymorphisms (SNPs), small deletions, and small insertions are inconspicuous aberrations in otherwise normal chromosomes that are often best elucidated with molecular techniques.

The addition of molecular technologies to traditional techniques such as the interpretation of hematoxylin and eosin- (H&E) stained sections, immunohistochemical staining patterns, flow cytometry data, and clinical information has revolutionized the practice of hematopathology. Improvements to the quality of patient care have resulted from the application of molecular methods to the diagnosis, prognosis, and treatment of hematologic disease.

☑ CHECKPOINT 42-1

Consider the role that oncogenes and tumor suppressor genes play in the development of cancer and the concept of personalized medicine.

OVERVIEW OF MOLECULAR TECHNOLOGIES

Clinical laboratories have experienced an influx of molecular technologies that were once mainly restricted to the research setting or to isolated molecular diagnostic laboratories. The initial molecular technologies that entered the clinical laboratory in the 1980s were labor intensive and required extensive molecular skills that the routine medical laboratory scientist did not possess. Our current understanding of the molecular basis of human pathology, including the identification of disease causing polymorphisms related to prognosis or therapeutic efficacy, has expanded the menu of molecular tests offered. Additionally, the spectrum of technology available to analyze genetic defects has expanded to include many user-friendly, semiautomated methods highly suitable for the clinical laboratory. Molecular testing can be used in hematology and hemostasis to assist in diagnosis, risk assessment, treatment optimization, and prevention of adverse drug reactions. In the future, health assessment and management applications can expand as evidence-based medicine research confirms the clinical utility of these applications.[1] Although many clinical laboratories do not offer an extensive menu of molecular testing, several technologies have become standard in the assessment of diseases of hematological and hemostasis origin.

Typical work flow in a molecular diagnostics laboratory begins with nucleic acid extraction (either DNA or RNA) and then assessment of quality and quantity followed by specific molecular techniques. These techniques can include variations of amplification, probe-based, and sequencing methodologies. A brief description of molecular methods follows.

Nucleic Acid Extraction

One important component of the molecular testing process is the isolation of nucleic acid, either DNA or RNA, by manual or automated methods. Various commercial extraction kits are available and have been expanded to automated extraction platforms. High-throughput molecular diagnostics laboratories require robotic systems to provide high-quality nucleic acids free from impurities and contamination. Several commercial platforms, including the Roche MagNA Pure LC system and the Qiagen Symphony, are able to provide high-quality DNA or RNA.[2,3] DNA can be isolated from whole blood, white blood cells, peripheral blood mononuclear cells, or cultured cells, and the resultant DNA is suitable for use in downstream reactions.[4]

Depending on the nature of the specimen, additional steps could be required to extract an optimum nucleic acid. For fresh or frozen tissue, homogenization must be performed. For formalin-fixed, paraffin-embedded (FFPE) tissue, paraffin removal could involve the use of an organic solvent such as xylene or a nonorganic solvent method available in a kit. DNA extracted from FFPE tissue has been cross-linked to proteins; therefore, high-molecular-weight DNA (intact or nondegraded DNA) is not available.[4] High-molecular-weight DNA is required when the particular target of interest (portion of the gene target) is large.

After nucleic acid extraction, either DNA or RNA requires an assessment of its purity and concentration. This can be achieved by spectrophotometrically measuring the intensity of absorbance of the nucleic acid solution at 260 nm and 280 nm. A pure sample of DNA has a 260:280 ratio of 1.8 and is relatively free from protein contamination. An RNA 260:280 ratio of ~2.0 indicates a pure preparation. Certain platforms (Agilent Bioanalyzer) have automated the determination of nucleic acid concentration and purity. Many molecular assays require high-quality DNA preparations, and others require high-quality RNA, for example, reverse transcriptase polymerase chain reaction (RT-PCR, described later in "Reverse-Transcriptase PCR [RT-PCR]").[4]

Nucleic Acid Amplification

Polymerase Chain Reaction (PCR)

At the core of molecular technology is the amplification technique called the **polymerase chain reaction** or **PCR**. This technique copies a particular segment of DNA one billion-fold. This process permits rapid, sensitive, and specific identification of a segment of DNA that can then be further tested for a disease-specific genetic defect.

The PCR technique amplifies (makes many copies of) a target sequence of DNA that lies between two regions of known sequence.

FIGURE 42-1 The structure of DNA. DNA is a double-stranded molecule composed of sequences of nucleotides. One strand is bound by hydrogen bonds (shown as diagonal bridges) to its complementary strand. According to the rules of complementary base pairing, the nucleotide adenine (A) is complementary to thymine (T), and guanine (G) is complementary to cytosine (C).

After double-stranded DNA is denatured, short oligonucleotide primers (pieces of DNA) anneal (bind) to complementary DNA sequences flanking the target. The oligonucleotides anneal based on Watson-Crick base pairing (A-T, C-G; Figure 42-1 ■). After primer annealing, the enzyme DNA polymerase extends the 3′ end of the primer using deoxynucleotides (dNTPs), including adenosine triphosphate (ATP), cytosine triphosphate (CTP), guanosine triphosphate (GTP), and thymidine triphosphate (TTP). Synthesis of the new DNA strand always occurs in the 5′ to 3′ direction because the new dNTP can be added only to the 3′ OH group. Under appropriate reaction conditions, new DNA that is complementary to the template strands is synthesized.[5]

Reagents for the PCR reaction include the template contained in the sample, a DNA polymerase, primers, dNTPs, $MgCl_2$, and buffer. One cycle of a PCR consists of DNA denaturation (95°C) and primer annealing (about 55–60°C), followed by extension (65–75°C). The process is repeated for 20–40 cycles in an instrument called a *thermocycler*. The repetition allows new DNA strands to be available to serve as template strands for subsequent cycles of amplification[5] (Figure 42-2 ■). After 32 PCR cycles, more than one billion copies of DNA have been generated.

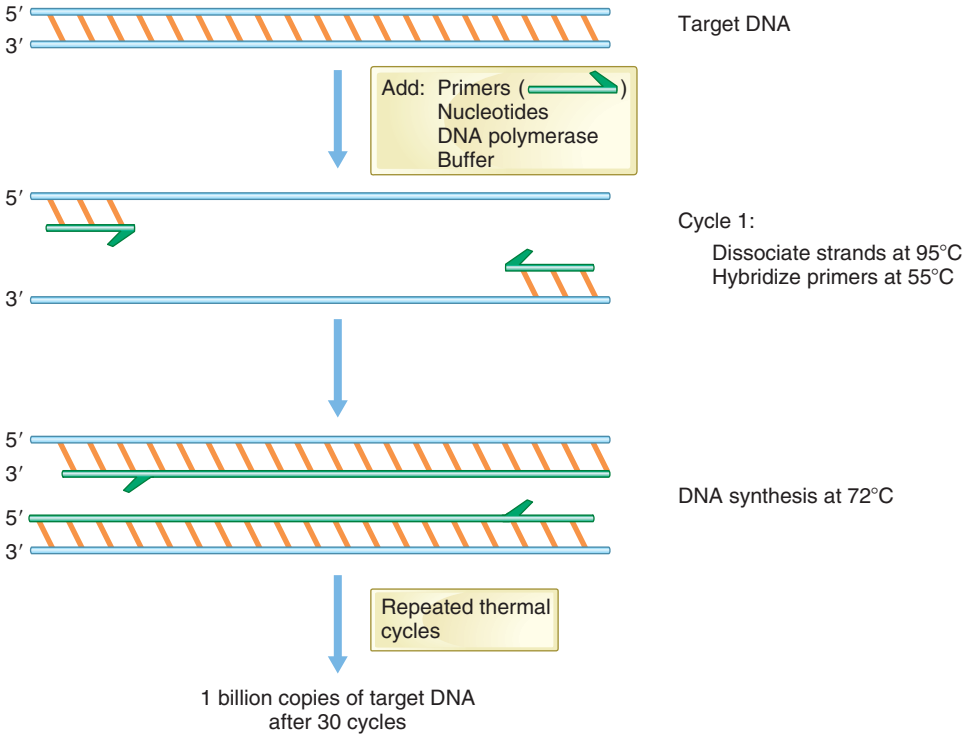
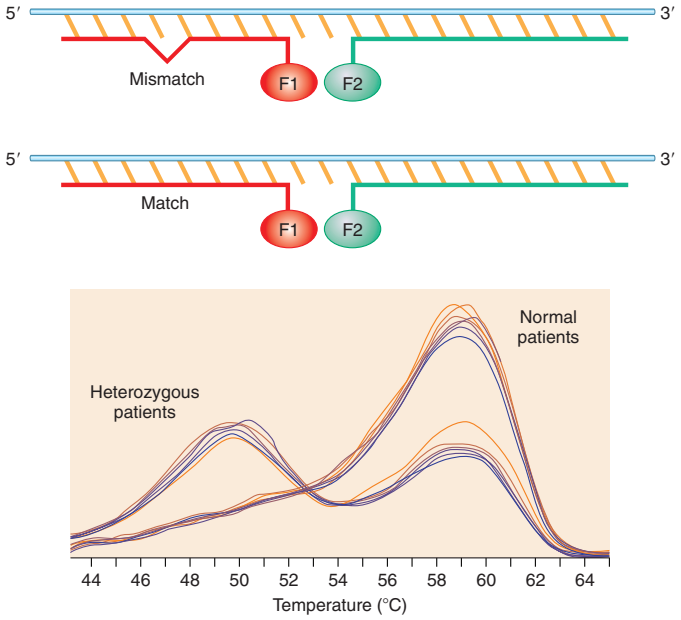


FIGURE 42-2 PCR is a method of enzymatically amplifying a particular segment of DNA through a process of repeated cycles of heating, cooling, and DNA synthesis. First the target (patient) DNA is mixed with the chemicals needed for DNA synthesis. Included are two short DNA probes (called *primers*, shown as half arrows) designed to flank the particular segment of DNA that needs to be amplified. A thermocycler instrument is programmed to sequentially heat and cool the sample. In cycle 1, the sample is heated to 95°C to dissociate complementary strands of DNA and then is cooled to 55°C to permit binding of the short DNA probes that serve as primers for subsequent enzymatic DNA replication at 72°C. This replication generates new complementary strands to produce an exact copy of the original target DNA. In subsequent cycles, the products of previous cycles can serve as templates for DNA replication, allowing an exponential accumulation of DNA copies. After 30 cycles, which takes only several hours, approximately 1 billion copies of the target DNA have been generated.

PCR = polymerase chain reaction

The products of the PCR reaction, or *amplicons*, are available for qualitative detection (presence or absence of the target) or further analysis. For traditional PCR reactions, products are frequently electrophoresed on an agarose gel and stained with ethidium bromide. A UV light source is used to visualize the DNA bands, which are compared with bands on standard DNA ladders to determine approximate size.[5] Other applications can include DNA sequencing of specific portions of the PCR product, high-resolution melt (HRM) analysis (Figure 42-3 ■), or **hybridization** techniques using a **probe** complementary to a specific DNA sequence.



■ **FIGURE 42-3** Melt curve analysis is a method used to determine whether a particular mutation is present by evaluating the temperature at which a labeled probe (shown in red) melts away from its target DNA. To accomplish this, a mixture of probe and target DNA is gradually heated until the probe melts away. Melting occurs at a low temperature if a mismatch exists but at a high temperature if there is a perfect match between the probe and target sequence. The temperature at which dissociation occurs is detected in a qPCR instrument that analyzes the interaction between a fluorochrome linked to the probe (shown in red) and another fluorochrome (shown in green) on an adjacent probe. Proximity of the red and green fluorochromes generates a yellowish signal when both probes are bound to the target, whereas the red and green fluorochromes floating apart indicate that melting has occurred. The graph depicts melt curves for a series of 20 patients who were tested for the *F2* mutation; those with two normal *F2* genes have a single melt peak at 60°C. In contrast, those patients having one mutant copy and one normal copy of the *F2* gene have two melt peaks, one at 50°C and the other at 60°C, indicating that they are heterozygous for the mutation and at increased risk for venous thrombosis.

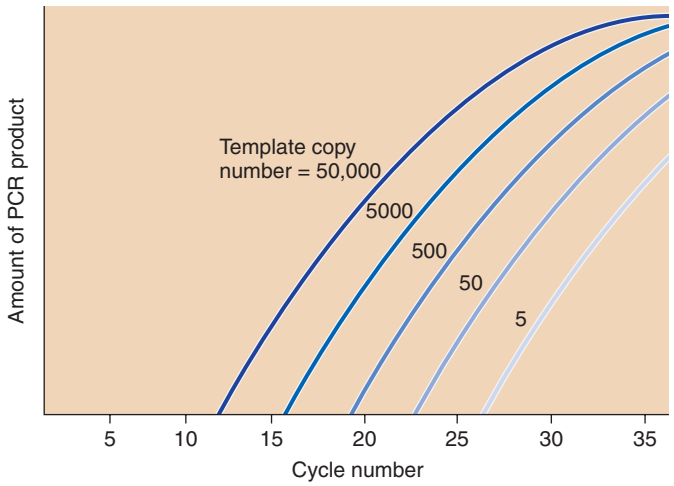
PCR = polymerase chain reaction; qPCR = real-time PCR

Real-Time Polymerase Chain Reaction (qPCR)

Traditional PCR can be used only to detect the presence of an amplicon but cannot be used to determine exactly how many copies have been generated. This limitation of the assay catalyzed the development of real-time PCR, or qPCR, that can be used to quantify the number of copies of a target sequence in a patient's sample. In addition, qPCR can be used to detect the mRNA product of DNA **transcription**. Importantly, qPCR is used in hematopathology to determine the proportion of mutated cells among normal cells in peripheral blood, otherwise known as *minimal residual disease*.[6]

Detection of qPCR products takes place during the exponential generation of amplicons (exponential phase). By detecting fluorescence in the exponential phase, a quantitative relationship exists between the amount (copy number) of the starting material in the sample and the cycle number at which the amplification curve crosses a mathematically determined threshold. An increase in a reporter—fluorescent signal—detected in the exponential phase is directly proportional to the number of amplicons generated (Figure 42-4 ■). By using a known series of diluted standards, a real-time instrument is able to perform a standard curve and extrapolate quantitative results for unknown samples by reporting copy number.[6]

Several detection technologies have been developed; SYBR Green® is the simplest. It attaches to double-stranded DNA in the minor groove and fluoresces. Therefore, when double-stranded PCR products are generated and SYBR Green® binds the DNA, the instrument software captures fluorescence. Other detection systems that provide more specific detection of the desired amplicon have been developed. One is the TaqMan system based on the 5′ exonuclease activity of DNA polymerase and captured fluorescence of a cleaved reporter dye-labeled probe.[7] TaqMan technology has been successfully incorporated into many clinical real-time protocols to provide sensitive and specific detection.



■ **FIGURE 42-4** Tumor burden in patients with APL can be measured using qPCR targeting PML/RARA transcripts. The higher the tumor burden, the more fusion transcripts are present, so the more template cDNA is present, and the earlier the products appear during PCR amplification cycles.

APL = acute promyelocytic leukemia; PCR = polymerase chain reaction

In a clinical setting, qPCR has advantages over traditional PCR for several reasons. The greatest advantage is that qPCR detection is accomplished while the PCR reaction takes place, eliminating the need for cumbersome, time-consuming, and nonautomated gel detection of traditional PCR. In addition, for gene expression studies, results can be expressed in fold differences by comparing the fluorescent signal generated by the sample to a signal generated by a reaction targeting a commonly expressed gene called a *housekeeping gene*.

The qPCR technology has revolutionized the practice of clinical molecular diagnostics by providing closed systems that are rapid and highly sensitive and can be customized to numerous applications. Among the many applications beyond hematopathology are viral quantitation of viral load for HIV-I and HCV, quantitation of gene expression (both absolute and relative), pathogen detection, microarray verification, genotyping, drug therapy efficacy, and quality control and assay verification.[7–9]

Reverse-Transcriptase PCR (RT-PCR)

The enzyme reverse transcriptase, whose gene is encoded by the HIV-I retroviral genome, can be used to produce a **complimentary DNA (cDNA)**, copy of an RNA sequence followed by a standard PCR to amplify the target sequence. RT-PCR is used when the target nucleic acid is RNA.[6] Applications include detection of RNA transcripts such as BCR/ABL1 (chronic myelogenous leukemia [CML]; Chapter 24) RUNX1-ETO (acute myeloid leukemia [AML]; Chapter 26), PML-RARα (acute promyelocytic leukemia [APL]; Chapter 26), and ETV6-RUNX1 (acute lymphoblastic leukemia [ALL]; Chapter 27).[6]

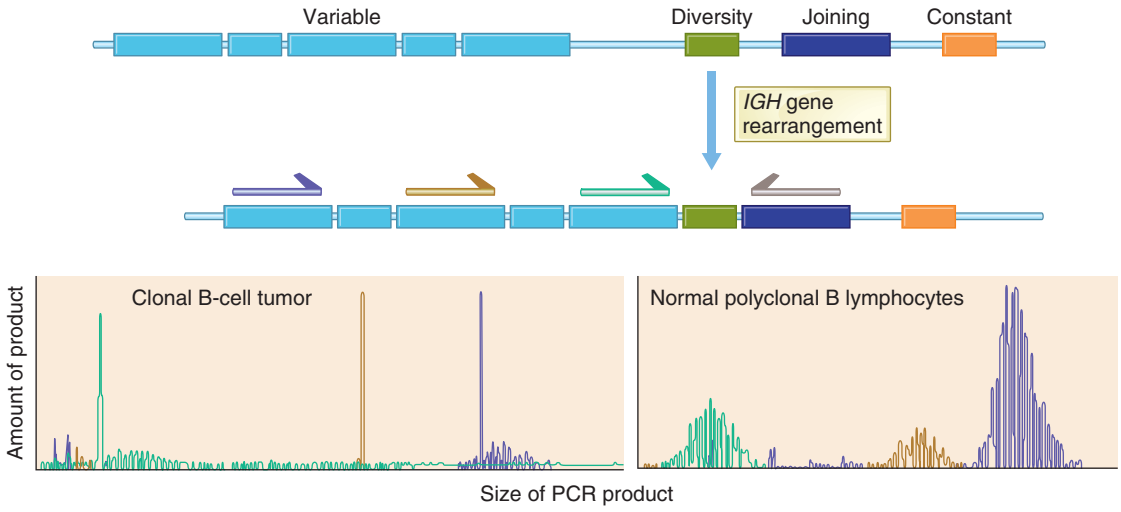
Multiplex Polymerase Chain Reaction (PCR)

Use of multiple sets of primers in one PCR reaction can simultaneously detect several targets.[6] Multiplex PCR can be used to detect multiple disease-associated mutations within one gene such as the numerous mutations in the β-globin gene of patients with β-thalassemia. It can also be used to detect B or T lymphocyte clonality of plasma cell malignancies. For example, when detecting and assessing minimal residual disease in a B-cell lymphoma, an immunoglobulin heavy chain (IGH) gene clonality assay can be used (Figure 42-5 ■). A typical IGH assay is based on multiplex reactions targeting conserved regions within the variable (V), diversity (D), and joining (J) regions of the immunoglobulin heavy chain. Finally, multiplex PCR can also be used in assays that identify multiple possible break points of translocations, for example, *BCR/ABL1, MLL/AFF1* (ALL; Chapter 27), and *NPM1/RARA* (APL; Chapter 26).[6]

Other Amplification Techniques

Numerous amplification technologies have been developed to circumvent patent obligations of PCR and offer selected advantages in particular applications. Among these variations are the transcription-based amplification systems exemplified by transcription-mediated amplification (TMA) by Genprobe and nucleic acid sequence-based amplification (NASBA) offered by Organon-Teknika. RNA serves as the typical target instead of DNA and the process is isothermal, not requiring a thermocycler. These processes have been used to amplify and quantify BCR/ABL1 transcripts in CML.[10]

Probe-based amplification methodologies amplify specific synthetic probes that hybridize to the target of interest rather than



■ **FIGURE 42-5** Rearrangement of the *IGH* gene involves random splicing of V, D, and J segments to produce a unique coding sequence. This process brings the V and J segments so close together that it becomes possible to PCR amplify across the rearranged gene by using several primers (shown as half arrows) targeting various V and J segments. In a B-cell tumor, all tumor cells contain exactly the same *IGH* rearrangement that was present in the original transformed B cell from which the tumor arose. This tumor-related clonal arrangement is identified by capillary gel electrophoresis as a spike (representing a single-size PCR product with each primer set). In contrast, benign tissue has normal B lymphocytes whose polyclonal rearrangements appear as multiple different-size PCR products.

V = variable; D = diversity; J = joining; PCR = polymerase chain reaction

amplifying the DNA target. Included in these methods are the ligase chain reaction (LCR) and strand displacement amplification (SDA). Sickle cell mutation analysis was one of the first applications of the LCR.[11]

Hybridization Techniques

Fluorescent in Situ Hybridization (FISH)

Fluorescent in situ hybridization (FISH) uses a labeled probe to detect and localize specific RNA or DNA sequences in tissue samples. FISH relies on DNA's ability to hybridize with a complimentary, fluorescently labeled nucleotide probe. *In situ* means "in the original place" in Latin. Ultimately, the location of the target sequence (DNA or RNA) can be detected in the cell, tissue, or chromosome. The sample is first fixed onto a glass slide and is treated with chemicals to permeabilize the cells and allow probe entry. If DNA is the target of interest, it must be denatured to make it single stranded so that the probe can hybridize. A fluorescently labeled nucleic acid probe complementary to the target of interest is added to the sample and excess probe is washed away. Fluorescence imaging is used to visualize the probe.

FISH is a particularly important technique for visualizing chromosomal translocations in leukemias (Table 42-1 ★). Allele-specific probes can be hybridized to the sample to reveal when two genes that should be found in separate, distinct locations, have been juxtaposed. For example, in normal cells, a probe that hybridizes to the *BCR* gene at 22q11.2 can be detected with a green fluorochrome, whereas the *ABL1* gene at 9q34 can be detected by a red fluorochrome. When translocated together, *BCR/ABL1* will appear as a bright yellow spot (the combination of green and red fluorochromes) in leukemia cells.[4]

Southern Blot

The **Southern blot** involves restriction digestion of sample DNA using **restriction endonucleases** followed by gel electrophoresis of the products (Figure 42-6 ■). Following denaturation of the double-stranded DNA, single-stranded DNA is then transferred or "blotted" to a more easily handled matrix, usually nitrocellulose or nylon. Capillary action of moistened filter paper facilitates the transfer of DNA to the new matrix. Ultraviolet light helps to secure the DNA to the membrane. Subsequently, a labeled probe designed to hybridize to the sequence of interest is incubated with the membrane-bound DNA, followed by a detection method specific to the type of label. The Southern blot has served as the gold standard to which newer and more rapid technologies have been compared. Although time consuming and technically difficult, the Southern blot can distinguish the single-base change found in patients with sickle cell anemia.[12]

Allele-Specific Oligonucleotides (ASO)

ASO technology, also called the *reverse dot blot*, amplifies, labels, and applies (blots) the patient's DNA in "engineered dots." The dots are constructed using short oligonucleotides (oligos) engineered to be complementary to known targeted sequences of WT or mutant alleles and fixed to a membrane for stability. The dot oligo hybridizes only to specific complementary sequences (blots) found in the patient's DNA. Numerous applications of this technology, involving known mutations, have been successful because the process can be completed in one day and is suitable for high-throughput laboratories. The ASO process has been successful for diseases characterized by a single mutation (sickle cell anemia) or only a limited number of mutations such as hereditary hemochromatosis. Reverse dot-blot analysis has been developed to screen many β-thalassemia mutations and to use in prenatal diagnosis.[12]

Microarray Technology

As the demand for high-throughput screening for gene expression has been accelerated by the quest for personalized medicine, the popularity of microarray technology has burgeoned. Microarray technology enables large-scale analysis of multiple targets simultaneously. A variety of microarray technologies exists, but in general, array targets are immobilized on a glass slide. The targets can be DNA, cDNA, PCR products, RNA, or proteins. These targets are spotted on a slide in triplicate, incubated with fluorophore-labeled patient sample, and read by a fluorometer.[13] A variety of vendors has evolved alternate detection chemistries.

★ **TABLE 42-1** Genetic Abnormalities of Neoplastic Hematological Disorders That Can Be Detected by FISH

Hematological Anomaly	Detection by FISH
AML	t(8;21), t(6;9)
APL	t(15;17) (PML-RARA)
AMML	t(11;21)
AMoL	t(9;11)
CML	t(9;22), t(11;22)
B-cell leukemia	t(2;8), t(8;14), t(8;22), t(11;14)
CLL	Deletions: 11q22 (ATM), 13q14 (DLEU1/2 and *RB1*) and 17p13 (p53) Duplications: 6q, trisomy 12
ALL	t(9;22), t(12;21), t(8;14)
Multiple myeloma	t(14q32)
Myelodysplastic syndrome	5q-

AML = acute myelogenous leukemia; APL = acute promyelocytic leukemia; AMML = acute myelomonocytic leukemia; AMoL = acute monocytic leukemia; ATM = ataxia telangiectasia mutated; CML = chronic myelocytic leukemia; CLL = chronic lymphocytic leukemia; DLEU1/2 = deleted in leukemia; RB1 = retinoblastoma.

Courtesy of Kristin Landis-Piwowar, Ph.D., MLS (ASCP), assistant professor, Biomedical Diagnostic and Therapeutic Sciences, School of Health Sciences, Oakland University.

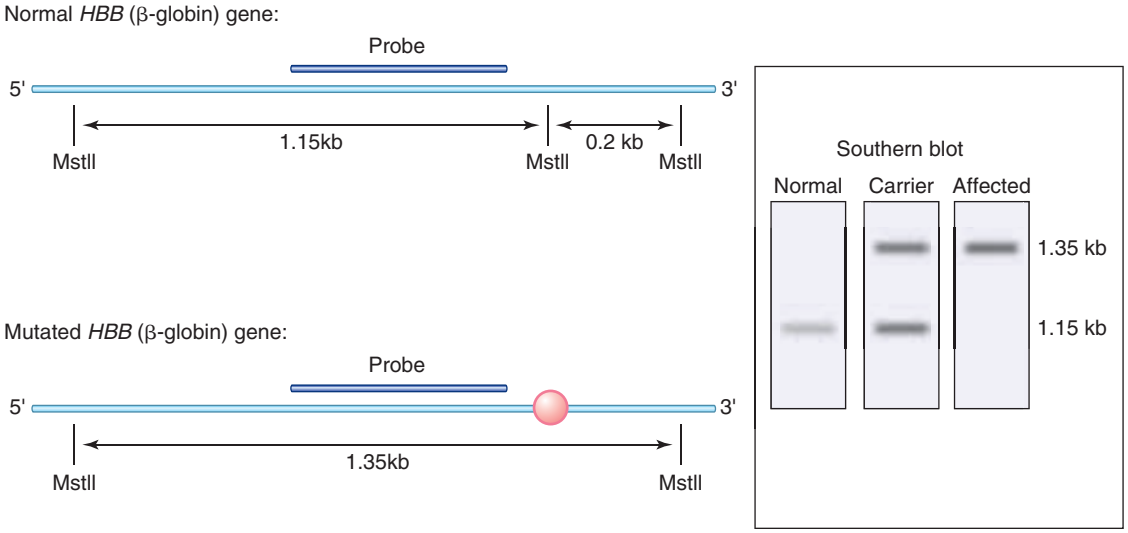


FIGURE 42-6 Southern blot analysis of the sickle cell mutation is accomplished by first extracting DNA and then cutting it with MstII restriction endonuclease that cleaves DNA at a specific nucleotide sequence (vertical lines). Because that specific sequence is present many times in the human genome, MstII cuts genomic DNA into many small fragments. The resultant DNA fragments are separated by size using gel electrophoresis, dissociated into single strands by soaking in an alkaline solution, and then transferred to a nylon membrane by a blotting procedure. To identify the fragment containing the *HBB* gene, the membrane is soaked in a radiolabeled DNA probe (bar) that hybridizes to a complementary segment of the *HBB* gene. The pattern of bands that the probe recognizes reflects the size of the corresponding restriction fragments measured in kilobases (kb). An *HBB* gene harboring the sickle mutation (shown as a pink circle) fails to cut with MstII, thus altering the band pattern. In this way, a person affected by sickle cell anemia can be distinguished from a carrier (sickle trait) and from a person of normal genotype. Note that restriction endonucleases are naturally occurring enzymes that recognize and cleave specific nucleotide sequences in DNA. Each restriction endonuclease is named for the bacteria from which it was purified. For example, EcoRI is derived from *Escherichia coli* and cleaves DNA having the sequence 5′-GAATTC-3′, whereas HindIII derived from *Haemophilus influenza* cleaves at 5′-AAGCTT-3′. In this example, MstII cleaves the normal DNA when it recognizes the sequence 5′-CCTGAGG-3′, but it cannot cleave DNA harboring the sickle mutation 5′-CCTGTGG-3′.

MstII = *Microcoleus* spp

One particular application of the microarray is for gene expression profiling. Clinicians can seek to determine which genes are being differentially expressed in leukemic cells compared with normal cells.[13]

Microarray technology can be used to classify ALL by analyzing gene expression patterns in leukemic cells and to identify expression of genes associated with prognosis or to identify candidate genes for new anticancer therapies.[14]

Bead Array Technology

A variation of microarray technology can be used with bead array techniques. Probes can be immobilized on beads to provide a surface for hybridization of amplified samples. Numerous samples can be tested simultaneously for a series of SNPs or **genotypes**. Fluorescent color coding beads and labeling the sample with an alternative fluorescent dye allow the hybridized combination to be detected. Software analysis provides interpretation of the patient results.[15] Luminex Corp. has been a pioneer in bead array technology with commercial systems adapted to genotyping for CYP2C19 related to clopidogrel (Plavix) metabolism.

CASE STUDY *(continued from page 888)*

After viewing the bone marrow differential and molecular results, a diagnosis of CML is made, and Warren is placed on imatinib mesylate therapy of 450 mg daily, which he tolerates well. After 3 months of treatment, Warren exhibits a complete hematologic response and a partial cytogenic response with 30% or 6 of 20 Ph+ metaphases (by FISH) and 7% International Scale (IS) by qPCR. Treatment is continued, and by his 12-month examination, Warren has a complete cytogenetic response of no Ph+ metaphases, and <0.1% IS. Imatinib treatment is discontinued, but Warren continues to see his physician every 6 months for follow up testing.

1. What type of molecular diagnostic testing would be suitable to follow Warren's minimal residual disease (MRD)?

Direct DNA Sequence Analysis

DNA sequencing is used to determine the nucleotide sequence in a segment of DNA by replicating the DNA strands and monitoring the order in which labeled nucleotides are added to the new strands. It is particularly useful for detecting point mutations located anywhere in the DNA segment.

Chain Termination Sequencing

Traditional DNA sequencing is based on the Sanger dideoxynucleotide chain termination method. This method involves the addition of an unusual dideoxyribonucleotide (ddNTP) that is missing the 3′-OH group necessary for nucleotide addition. The ddNTPs ultimately determine the sequence of a target DNA template as they are incorporated into a growing DNA strand that is complementary to the target sequence.[4,6]

Current sequencing technologies can be based on the chain termination methodology and have been adapted to an automated format. The automated instrument detects four fluorescent dyes with distinct but slightly overlapping wavelengths that are attached to the ddNTPs. The instrument produces a series of peaks called an *electropherogram* that correspond to the four bases (A, T, C, G). The software assigns a letter to the corresponding dye peak as that ddNTP is incorporated into the growing strand of DNA that is complementary to the template. Clinical analysis of the sequence information is incorporated into the instrument software and detailed reports provide both the sequence data and interpretation relative to known mutations or SNPs that are implicated in disease. Numerous software programs can be used initially to develop interpretative clinical reports comparing patient sequences with known variations related to pathology.[4] For a video of DNA sequencing, go to this chapter's Companion Resources.

Next Generation

Next-generation sequencing technologies have been developed to provide sequencing information for large numbers of templates and provide coverage over whole genomes with single-base pair resolution.[4] Financial pressures have driven the development of high-throughput sequencing methods that produce thousands of sequences in parallel. Costs for next-generation sequencing are lower than with the traditional dye termination methods. Numerous vendors offer next-generation sequencing systems including Applied Biosystems' SOLID system, Illumina's HiSeq systems, Life Technologies Ion Torrent™ Semiconductor Sequencing Chips, and Roche's GS FLX+ system. They can differ in output, run time, read length, and accuracy. One system may be more suited for a particular genomic sequencing application than another. Next-generation sequencing has been used to identify novel mutations in disorders such as T-ALL, AML, and CML.[6]

CASE STUDY (continued from page 894)

After 3 years of follow-up testing to track his MRD, Warren's qPCR reveals an increase in IS to 4%. This is worrisome because it suggests that he is coming out of remission. Imatinib mesylate therapy is resumed, but after 2 months of treatment, Warren's IS had risen to 10%, indicating a resistance to imatinib treatment.

Several possible point mutations in *BCR/ABL1* can result in imatinib resistance.

2. Before increasing Warren's imatinib dose, what type of molecular testing might Warren's physician order to help in the decision about how to manage Warren's disease?

CLINICAL APPLICATIONS OF MOLECULAR DIAGNOSTICS IN HEMATOPATHOLOGY

Molecular methods are increasingly valuable for prognostication, therapeutic response to treatment, and detection of minimal residual disease in hematopathology. Genetic abnormalities can be used for predictive prognostic information about a disease when they appear either by themselves or when present with other genetic mutations (e.g., *NPM1* mutations are associated with a good prognosis in the absence of *FLT3* internal tandem duplication [ITD] mutations, but they confer an intermediate prognosis if they are present in conjunction with *FLT3*ITD[15]) (Chapter 26).

Mutation analysis also impacts treatment decisions. Tyrosine kinase inhibitors (TKIs) have been used successfully to treat malignancies, and they are examples of the use of gene mutations as potential therapeutic targets. Malignancies in which the receptor tyrosine kinase KIT has a gain of function can respond favorably to TKIs, although the strength of the response depends on the type of KIT mutation that is present.[14]

Finally, the presence or absence of MRD has a direct impact on prognosis, evaluation of relapse, and decisions concerning further treatment. Amplification technologies are especially sensitive in detecting MRD because as few as 0.01% of abnormal cells carrying a genetic abnormality can be detected. Moreover, molecular responses can be associated with disease outcome. For example, detection of *BCR/ABL1* is diagnostic of CML, and a reduction of *BCR/ABL1* by 3 log or more is sufficient for an excellent prognosis, whereas a rise in *BCR/ABL1* expression heralds the reappearance of CML.[16]

Likely, the greatest value of hematopathology is the use of molecular technologies for diagnosis of disease. In the past decade, the number of identified genetic abnormalities that can serve as biomarkers for hematological disease has grown explosively. Identification of new biomarkers will undoubtedly continue to expand rapidly. It is important to note, however, that molecular testing is not always straightforward. For example, only about one-half of the patients with hemophilia A display an identifiable mutation in the gene encoding factor VIII. The other half presumably harbor mutations in other genes that result in abnormal proteins whose downstream effect is to diminish the expression of viable factor VIII protein.

Refer to Table 42-2 ★ for a list of some molecular tests used in clinical laboratories. Included here is a summary of some of the hematopathologies for which molecular testing is available to assist the clinician with diagnosis, prognosis, and therapeutic decision making. The list of diagnostic and prognostic factors is growing rapidly, making it difficult to determine which markers provide the most beneficial information. It is likely that an integrated testing algorithm will emerge.

★ TABLE 42-2 Molecular Testing in Hematopathologies

Hematopathology	Gene/Mutation/Location	Molecular Technique	Notes
Erythrocyte hematopathologies			
Fanconi anemia	*FANCA, FANCC, FANCE*, or *FANCG* (various chromosomes)	Amplification	
Hemochromatosis	*HFE* (6p22.2)	Amplification	Increases total body iron content; provides valuable diagnostic, prognostic information
Hereditary persistence of fetal hemoglobin	*HBB, HBG1, HBG2* on chromosome 11	Southern blot, multiplex PCR	
Thalassemia	α and β gene deletions/mutations on chromosomes 16 and 11	Amplification, sequencing, reverse dot-blot	Emphasis is on screening programs to detect thalassemia carriers in areas of high prevalence and in certain populations
Hemoglobinopathy	Point mutations on *HBB* resulting in hemoglobin S/C/E or *HBD/HBB* fusion resulting in hemoglobin Lepore on chromosome 11	Amplification, sequencing, multiplex PCR	
Polycythemia vera	*JAK2* (V617F) (9p24)	Amplification, HRM	
Leukocyte hematopathologies			
AML	*CEBP*α (19q13.1)	Amplification, HRM, sequencing	Various *CEBP*α mutations confer variable likelihood of achieving complete remissions; valuable in determining therapy
	NPM1 (5q35)	Sequencing, HRM, heteroduplex analysis	Characterization of *NPM1* mutations provides substantial prognostic value
	KIT (4q11-q12)	Sequencing, ASO, HRM, qPCR	Mutations are gain of function to a cellular tyrosine kinase receptor that culminates in a proliferation advantage for the cell
AML and ALL	*FLT3* (13q12)	Amplification	Internal tandem duplication/point mutations result in constitutive tyrosine kinase receptor activity; increases cell proliferation
Myeloproliferative neoplasms	*JAK2* (V617F) (9p24)	Amplification, sequencing, ASO, HRM, qPCR	Mutation characteristic of polycythemia vera; also seen in essential thrombocythemia and primary myelofibrosis
	MPL (1p34)	Amplification, sequencing, HRM, qPCR	
ALL, AML, MDS, plasma cell neoplasms	*NRAS/KRAS* (1p13.2/12p12.1)	Sequencing	Activating mutations lead to proliferation advantages for the affected cell
CML and ALL	*BCR/ABL1* t(9;22)(q34;q11.2)	FISH, sequencing, ASO	Fusion gene confers proliferation advantage to affected cells; mutations provide information concerning TKI therapy
Follicular lymphoma and diffuse large B lymphoma	*IGH/BCL2* t(14;18)(q32;q21)	FISH, qPCR, microarray	Leads to overexpressed BCL-2 protein and reduced apoptosis in cells
Mantle cell lymphoma	*CCND1/IGH* t(11,14)(q13;q32)	FISH	Dysregulated cell-cycle progression as gene for cyclin D is brought under control of *IGH* promoter
Burkitt lymphoma	*MYC/IGH* t(8;14)(q24.1;q32)	FISH	Dysregulated cell growth as *C-MYC* is brought under control of the *IGH* promoter
Acute promyelocytic leukemia	*PML/RARA* t(15;17)(q22;q12)	RT-PCR, FISH	*PML/RARA* rearrangement is important in APL because it is correlated with responsiveness to treatment with all-trans retinoic acid
Childhood ALL	*ETV6/RUNX1* t(12;21)(p13;q22).	Amplification	Fusion gene is associated with a favorable prognosis and helps determine treatment
Hemostasis disorders			
Warfarin response	*CYP2C9* on chromosome 10	qPCR, bead array	
Warfarin sensitivity	*VKORC1* on chromosome 16	qPCR	
Factor V Leiden	*F5, G1691A* SNP on chromosome 1	Amplification	

Hematopathology	Gene/Mutation/Location	Molecular Technique	Notes
Prothrombin G20210A	*F2*, G20210 SNP on chromosome 11	Amplification	Inheritance of both G1691A and G20210A confers at least a 20-fold increase in risk of a venous thromboembolic event
Hemophilia A/B	*F8/F9* >1000 mutations on X chromosome	Amplification, sequencing	
Methylenetetrahydrofolate reductase deficiency	*MTHFR*, C677T, and A1298C SNPs on chromosome 1	Amplification	SNPs result in reduced MTHFR enzyme leading to elevated homocysteine; even small elevations greatly increase thrombotic risk
Von Willebrand disease	*VWF* on chromosome 12		Due to complexity of the VWD genotype, VWD diagnosis no longer includes von Willebrand factor (*VWF*) gene mutations

ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia; APL = acute promyelocytic leukemia; ASO = allele-specific oligonucleotide; CML = chronic myelogenous leukemia; FISH = fluorescent in situ hybridization; HRM = high-resolution melt curve analysis; MDS = myelodysplastic syndrome; qPCR = quantitative (real-time) PCR; RT-PCR = reverse transcriptase-PCR; SNP = single nucleotide polymorphism; TKI = tyrosine kinase inhibitor

☑ CHECKPOINT 42-2

List and briefly describe the four general ways in which molecular diagnostics is useful to clinicians in delivering care to their patients.

Erythrocyte Disorders

Most erythrocyte disorders for which molecular testing exists are inherited disorders. A rapidly expanding list of molecular testing for erythrocyte disorders is emerging in the clinical laboratory today. Whereas traditional diagnostic methods are sometimes still preferred, molecular testing can provide reliable information that aids in the diagnosis of several erythrocytic diseases. Erythrocyte disorders in which molecular testing has been useful but are not listed here include hemolytic anemias with membrane structural defects or enzyme deficiencies and porphyrias.

Fanconi Anemia (FA)

More than 15 genes are implicated in the development of FA (Chapter 16); however, most people have a mutation in the *FANCA, FANCC, FANCE*, or *FANCG* genes.[17] Molecular genetic testing with amplification methodologies is clinically available for most of the genes, but diepoxybutane-induced chromosome breakage remains the gold standard of diagnosis.

Hemochromatosis (HFE)

Mutations in the *HFE* gene are seen in *HFE*-associated hereditary hemochromatosis (Chapter 12). Previously, a liver biopsy was the gold standard for diagnosis, but more recently, less invasive molecular testing, such as amplification methods, is gaining favor with clinicians.[18]

Hereditary Persistence of Fetal Hemoglobin (HPFH)

HPFH (Chapter14) can result from large deletions of promoters of the γ-globin genes *HBG1* and *HBG2*. Southern hybridization and multiplex PCR are used to identify the mutations.[19]

Thalassemia

The molecular basis of thalassemia lies in mutations that alter the genes encoding either α- or β-chains that comprise hemoglobin (Chapter14). Nonmolecular testing (mean cell volume/red blood cell count ratio and hemoglobin electrophoresis) continues to be very useful in screening for thalassemias. Molecular techniques that utilize amplification are cost and time effective, although sequencing remains the preferred method for second-line testing.[20]

Hemoglobinopathies

Hemoglobin (Hgb) S, Hgb C, and Hgb E are abnormal variations of Hgb A and account for a large percent of hemoglobinopathies that develop as a result of amino acid substitutions in the β-globin chain of hemoglobin (Chapter 13). Although the abnormal proteins can be identified with protein electrophoresis, DNA amplification and sequencing of the β-globin gene cluster provide reliable testing methodologies.[21]

Hgb Lepore is a rare hemoglobinopathy in which a fusion occurs between the δ-globin and the β-globin genes, *HBD* and *HBB*.[22] Multiplex PCR and gel electrophoresis can be used to detect the three common Hgb Lepore mutations.

Polycythemia Vera

A somatic mutation in *Janus kinase 2* (*JAK2*) at exon 14 causes the V617F mutation, which is found in about 97% of patients with polycythemia vera (Chapter 24). Although the single SNP mutation in exon 14 is the most common mutation that results in polycythemia vera, many cases have a *JAK2* exon 12 mutation. These mutations are revealed with PCR amplification techniques and HRM analysis.[23]

Leukemia

Cytogenetic analysis or karyotyping is essential for diagnostic evaluation of hematopathologies, especially the leukemias (Chapter 41). Many leukemic disorders result from somatic mutations in either a multipotential or lineage-restricted progenitor. Cell signaling and downstream growth, differentiation, and proliferation become dysregulated as a result (Chapter 23).

Cytogenetic analysis can reveal chromosomal deviance such as translocations, insertions, inversions, deletions, and repeats that are strong predictors of leukemia. Molecular DNA testing can reveal these same genetic abnormalities by designing nucleic acid probes that recognize the abnormal fusion gene. Although DNA technology is more sensitive than karyotyping, it is limited to testing for a specific gene mutation. On the other hand, karyotyping has the advantage of presenting the clinician a global picture of the patient's entire genetic situation. The following sections provide a compilation of molecular abnormalities and examples of the genes commonly involved in leukemia.

Molecular Abnormality: Deletions or Insertions

CEBPα

CEBPα encodes a transcription factor that is a key regulator of myeloid proliferation and granulocyte/monocyte differentiation (Chapter 26). Mutations to *CEBPα* occur in 5–15% of AML patients.[24] Mutations are of two types: one mutation results in a lack of an essential activation domain and the other alters the protein's ability to bind to DNA. *CEBPα* mutation analysis could be valuable in determining therapy because certain *CEBPα* mutations confer a better likelihood of achieving complete remission than others. Suitable methods of *CEBPα* mutation detection include amplification techniques sequencing, and HRM analysis followed by sequencing.

FLT3

FLT3 encodes a cell surface tyrosine kinase receptor that is expressed on early myelo- and lymphopoietic progenitors (Chapter 26). Two mutations are frequently seen in AML. These mutations can present with either a normal karyotype or with the characteristic karyotype of APL, t(15,17). The most prevalent mutation is an ITD found in exon 14 through part of exon 15.[14] The size of the duplication is quite variable, from 3 nucleotides to >400, but in any event, the receptor's tyrosine kinase domain (TKD) is left intact. The other mutation is less common; it is a missense mutation caused by a single codon deletion in a portion of the gene coding for the TKD. Either mutation results in constitutive TKD activity leading to aberrant growth and proliferation, blocked differentiation, and a worse prognosis for the patient.[14] *FLT3* ITDs can be detected by PCR followed by analysis of the length of the amplicon because ITDs produce abnormally large amplicons.[14]

NPM1

NPM1 is a proto-oncogene and tumor suppressor gene that encodes a multifunctional protein, nucleophosmin. Approximately one-third of AML patients exhibit mutations in the *NPM1* gene (Chapter 26). Generally, these patients present with a normal karyotype, internal tandem duplication in the *FLT3* gene, a relatively higher WBC count in the peripheral blood, a high bone marrow blast count, and a lack of CD34 antigen.[25,26] *NPM1* mutations with an insertion into exon 12 cause a frame shift that disrupts the nuclear localization signal and results in abnormal cytoplasmic accumulation of nucleophosmin protein. Characterization of *NPM1* mutations provides substantial prognostic value; patients with them are significantly more likely to achieve a complete response to standard chemotherapy, especially if they lack *FLT3* mutations.[27] Direct sequencing, HRM, and numerous other molecular techniques are used to detect *NPM1* mutations. Immunohistochemistry can be used to reveal the cytoplasmic location of nucleophosmin, in which case an *NPM1* mutation can be assumed.

Molecular Abnormality: Point Mutations and Small Insertions/Deletions

KIT

KIT encodes a tyrosine kinase receptor (Chapter 24). The most frequent *KIT* mutations are point mutations and insertions/deletions. The mutations are gain of function and result in constitutive activation that culminates in a proliferation advantage for the cell. Detection of *KIT* mutations is important for diagnosis, prognostication, and therapeutic strategy (i.e., determination of the suitability of TKIs) of myeloproliferative neoplasms because drug responsiveness appears to be closely correlated with mutation type. *KIT* mutations are detected using direct sequencing, ASO, HRM, and qPCR.[28]

Janus Kinase 2 (*JAK2*)

JAK2 encodes a nonreceptor protein tyrosine kinase that participates in signal transduction from a variety of receptors (Chapter 24). These include two receptors that participate in signal transduction essential for hematopoietic development, the erythropoietin and thrombopoietin receptors. A somatic mutation in *JAK2* (1849G>T) causes the *JAK2*(V617F) mutation that is found in about 97% of patients with polycythemia vera and in 50% of all patients presenting with essential thrombocythemia and primary myelofibrosis.[29] A variety of molecular techniques, including DNA sequencing, ASO, HRM, and qPCR, can detect *JAK2* mutations.[30]

Myeloproliferative Leukemia Virus (MPL) Oncogene

The *MPL* encodes the thrombopoietin receptor. Ligand binding to this receptor promotes megakaryopoiesis (Chapter 24). Somatic mutations resulting in a W515L substitution in the MPL protein are the most frequently noted mutation, but mutations of W515K and W515S have also been observed. These mutations are most often seen in essential thrombocythemia and primary myelofibrosis.[30] Some patients have been determined to have a concurrent *JAK2* (V617F) mutation, the one commonly associated with PV. Mutation detection is important for diagnostic purposes, but the prognostic implications are uncertain. Direct sequencing, PCR followed by HRM, and qPCR have all been used to detect *MPL* mutations.

NRAS/KRAS

NRAS/KRAS mutations often underlie disrupted cell signaling (RAF-MEK-ERK signaling) and produce a proliferation advantage in affected cells. *NRAS* (G12V) and *KRAS* (G12D) mutations are found in myelodysplastic syndromes (Chapter 25). Mutations in these genes are also seen in AML, ALL, multiple myeloma, and plasma cell leukemia.[31] Sequence analysis is a commonly used technique for mutational analysis.

☑ CHECKPOINT 42-3

NRAS/KRAS mutations are seen in a wide variety of hematological cancers including ALL, AML, and plasma cell neoplasms. Explain how mutations in these genes contribute to the etiology of these diseases.

Molecular Abnormality: Translocation

The six translocations described here are some examples of the most commonly encountered translocations in leukemia/lymphoma. It is important to note that individual tumors can be characterized by heterogeneity.

BCR/ABL1

The t(9;22)(q34;q11.2)*BCR/ABL1* mutation provides valuable information for decisions concerning therapy in CML and Philadelphia chromosome + ALL (Chapter 24). Mutations in the TKD of the *BCR/ABL1* fusion gene confer resistance to the prototypical TKI, imatinib, used to treat both pathologies.[31] Alternative TKIs can be beneficial in these patients. FISH, direct sequencing of PCR amplified products, and ASO are used to detect mutations.[31]

IGH/BCL2

The t(14;18)(q32;q21)*IGH/BCL2* is the mutation associated with follicular lymphoma (Chapter 28) in which the antiapoptotic gene *BCL2* is brought under the control of the *IGH* promoter. The influence of Ig heavy chain promoter leads to overexpression of the BCL2 protein and reduced apoptotic cell death in cells with this mutation. *IGH/BCL2* can be detected by FISH, qPCR, and microarray analysis.[32]

CCND1/IGH

The t(11,14)(q13;q32)*CCND1/IGH* mutation is found in nearly all mantle cell lymphomas. Translocation places the *CCND1* (cyclin D) gene next to the *IGH* promoter. This genetic change triggers the transformed B lymphocyte (lymphoma cell) to overproduce cyclin D1, a protein that directs progression of cells from G_1 to S phase of the cell cycle (Chapters 23, 28). FISH is often the method of choice to detect this translocation (Chapter 40).

MYC/IGH

The proto-oncogene *MYC* (*C-MYC*) encodes a nuclear protein involved in nucleic acid metabolism and mediating the cellular response to growth factors. The t(8;14)(q24.1;q32) rearrangement leads to dysregulated cell growth because *C-MYC* is brought under control of the promoter for IGH in lymphoproliferative disorders (Chapters 27, 28). The variability in the translocation break point in this **gene rearrangement** makes FISH a good choice for detection.

PML/RARA

In APL, the *PML* gene is juxtaposed with the retinoic acid receptor α (*RARA*) gene by the translocation t(15;17)(q22;q12). Three *PML/RARA* translocation patterns have been identified and the rearrangement is important in APL because it correlates with responsiveness to treatment with all-trans retinoic acid (ATRA) or arsenic therapy (Chapter 26). The rearranged DNA fragment can also be used as a specific diagnostic marker to detect MRD following treatment.[33] RT-PCR and FISH are commonly used to detect *PML/RARA*.

ETV6(TEL)/RUNX1(AML1)

The t(12;21)(p13;q22) translocation fuses the two genes *ETV6* and *RUNX1* and is the most frequent gene fusion in childhood ALL (Chapter 27). Unlike other ALL-associated chromosomal translocations, *ETV6/RUNX1* is associated with a favorable prognosis, and its identification helps the clinician select an appropriate chemotherapy regimen. Eradication of cells carrying the aberrant gene is expected in >90% of ALL cases with appropriate therapy.[34] The fusion gene is undetectable by conventional cytogenetic analysis, but PCR amplification across the translocation is an established methodology.

★ **TABLE 42-3** Pathogens of Hematologic Significance Detectable by Molecular Techniques

Cytomegalovirus (CMV)
Epstein-Barr virus (EBV)
Human herpes virus 8 (HHV8)
Human immunodeficiency virus (HIV)
Human T lymphotropic virus type 1 (HTLV1)
Malaria
Mycobacteria
Mycoplasma
Parvovirus B19
Toxoplasma

Infectious Diseases

Molecular testing for the presence of infectious disease has an application for hematological pathologies (Table 42-3 ★). Three of the pathogens listed, human T lymphotropic virus type 1 (HTLV1), Epstein-Barr virus (EBV), and human herpes virus 8 (HHV8) have been consistently linked to lymphoid neoplasms. About 0.1% of people infected with HTLV1 eventually develop adult T-cell leukemia/lymphoma. EBV is the causative agent of infectious mononucleosis and is believed to be an etiologic agent in the development of Burkitt lymphoma as well as immunodeficiency-related lymphomas and many Hodgkin lymphomas. HHV8 has been isolated from a majority of Karposi sarcomas in AIDS patients and can cause other tumors such as primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD).[35]

Molecular technology has provided new tools for detecting microorganisms based on the unique genetic code of each species. DNA amplification strategies are most useful because they are sensitive, specific, and rapid for detecting pathogen-specific nucleic acid sequences. Quantitative qPCR assays are useful for monitoring the level of organisms during treatment. In situ hybridization is helpful for identifying lesion-specific pathogens in biopsy samples.

CLINICAL APPLICATIONS OF MOLECULAR DIAGNOSTICS IN HEMOSTASIS

Molecular testing in disorders of hemostasis is proving to be invaluable for diagnosing hemostasis disorders, screening potential carriers of hemostasis diseases, and making therapeutic decisions. It is beyond the scope of this chapter to provide a comprehensive review of all genetic abnormalities that underlie disorders of hemostasis, but a review of some of the most commonly encountered molecular aberrations follows.

CYP2C9

CYP2C9 is a cytochrome P450 enzyme that is involved in the metabolism of several medications. CYP2C9 metabolizes >100 therapeutic drugs including warfarin, a widely prescribed anticoagulant, but

its dosage is difficult to predict because of widely variant patient responses. *CYP2C9* genotype establishes a patient's ability to metabolize this medication. For example, individuals with the *CYP2C9*2* or *CYP2C9*3* genotypes show reduced ability to metabolize warfarin, leading to its prolonged retention in their system. These patients require a reduced dose of warfarin for safe anticlotting treatment. qPCR is used to determine *CYP2C9* polymorphisms.[36]

VKORC1

This gene's product encodes the enzyme that is responsible for reducing vitamin K 2,3-epoxide to an active form so that it can participate in the synthesis of several vitamin K-dependent coagulation factors. Fatal bleeding can be caused by vitamin K deficiency and by warfarin therapy because warfarin is a vitamin K antagonist. Mutations in this gene are associated with deficiencies in vitamin K-dependent clotting factors. Patients harboring this mutation should be given a reduced warfarin dose. As with CYP2C9, molecular testing is most often by qPCR.[36]

Factor V Leiden (FVL)

A lack of standard nomenclature means that the SNP that is responsible for the factor V (*F5)* mutation responsible for the development of FVL can be referred to in several ways, including G1691A, Arg-506Gln, and R506Q. Although clotting assays have traditionally been used to diagnose this disorder, molecular testing has the advantage of determining whether patients are homozygous or heterozygous for the mutation. Homozygotes have more than 10 times the clotting risk of heterozygotes. Molecular testing includes PCR amplification.[37]

Prothrombin G20210A

Base substitutions in the prothrombin (*F2*) gene represent the second most prevalent hereditary defect underlying thrombosis risk. An SNP is responsible for a gain of function mutation that leads to excessive plasma prothrombin. Inheritance of both FVL and G20210A confers a significant increase in risk of a venous thromboembolic event. PCR is used to detect these mutations.[37]

Hemophilia A

Hemophilia A can arise from numerous mutations, including inversions, insertions, and deletions of the factor VIII (*F8*) gene. Carrier testing on daughters of either hemophiliacs or carriers and prenatal testing of a fetus can be performed with amplification methods.[38]

Hemophilia B

More than 1000 mutations in the factor IX gene have been reported. Carrier status of daughters of men with hemophilia B and at risk fetuses is commonly achieved using amplification techniques. Direct sequencing of the factor IX (*F9*) gene is available at large reference laboratories.[38]

Methylenetetrahydrofolate Reductase (*MTHFR*)

A C>T missense mutation in the *MTHFR* gene results in the substitution of alanine for valine with the downstream effect of elevated levels of plasma homocysteine. Even small increases in homocysteine are attributed to increased thrombotic risk. Molecular testing for the *MTHFR* mutation has become readily available in the clinical laboratory. The most common methodologies are PCR based.

von Willebrand Disease (VWD)

Due to the complexity of VWD genotype, the criteria for its diagnosis no longer include von Willebrand factor (*VWF*) gene mutations. The International Society of Thrombosis and Hemostasis maintains a database of VWF polymorphisms.[39]

☑ CHECKPOINT 42-4

Explain how PCR technology can provide quantitative results. Suggest several hematologic and hemostasis applications of qPCR technology.

Summary

DNA technology provides a powerful new tool for laboratory diagnosis of a wide variety of hematologic diseases including inherited diseases, infectious diseases, and cancer. Many of the erythrocytic and hemostatic diseases that are amenable to molecular diagnosis are inherited diseases whose mutations and genetic aberrations are discernable with multiple molecular techniques. Leukemias and lymphomas have chromosomal translocations or clonal gene rearrangements that are also easily confirmed with molecular diagnosis. All infectious organisms are suitable targets for molecular detection because invading organisms have unique genomes that can be differentiated from the host genome, denoting infection.

The molecular methods commonly used include multiple variations of nucleic acid amplification, fluorescent in situ hybridization, and Southern blot analysis. Newer methods such as DNA sequencing and microarray technology are also gaining a foothold in clinical laboratory testing.

Review Questions

Level II

Use this case history for questions 1 and 2.

A 38-year-old woman complained of dizziness, fatigue, and abdominal pain. On physical examination she appeared pale, and her spleen was enlarged. Laboratory studies revealed anemia and an elevated leukocyte count of 69×10^9/L. A complete spectrum of granulocytic cells from myeloblasts to neutrophils was present in the blood, and the number of basophils was increased. The LAP score was low. The bone marrow could not be aspirated, but a biopsy revealed that the marrow was packed with myeloid elements. Cytogenetics could not be performed because of the lack of an adequate marrow aspirate and the inability to induce cell division in peripheral blood leukocytes. Blood was submitted for molecular diagnostic testing.

1. Which genetic defect is the most appropriate target for molecular testing to assist in diagnosing the patient's hematologic disorder? (Objective 4)
   A. *PML/RARA*
   B. *BCL2/IGH*
   C. *BCR/ABL1*
   D. *BCL1/IGH*
2. Is there any reason to use molecular testing for the patient at a later date? (Objectives 3, 6)
   A. No, the positive test results are definitive, and nothing more can be accomplished.
   B. Yes, the patient can be monitored to detect minimal residual disease following therapy.
   C. No, but family members should be tested for the same molecular defect.
   D. Yes, the translocation break point must be sequenced to prove which genes are involved.
3. Which of the following reagents is most critical for making a PCR specific for the *F5* gene mutation (*FVL*) as opposed to a prothrombin *F2* gene mutation? (Objective 2)
   A. Primers
   B. Nucleotides
   C. DNA polymerase
   D. Buffer
4. All molecular tests that analyze specific portions of the human genome rely on the principle that: (Objective 2)
   A. DNA is different in every cell of a particular individual.
   B. Probes bind to their complementary target sequence through the hybridization process.
   C. Restriction endonucleases cut sites that remain the same regardless of any mutations.
   D. Heat or alkaline pH can convert single-stranded DNA to double-stranded DNA.
5. Which of the following assays is most appropriate for detecting a tumor-associated genetic defect that is present in only 0.1% of the cells in a patient sample? (Objective 7)
   A. PCR
   B. Southern blot analysis
   C. karyotyping
   D. immunophenotyping
6. PCR differs from reverse transcriptase PCR (RT-PCR) in the following way(s): (Objectives 2, 7)
   A. Ribonucleotides rather than deoxyribonucleotides are added to the reaction mixture of RT-PCR.
   B. Following amplification, PCR generates a DNA product, whereas RT-PCR generates an RNA product.
   C. RNA rather than DNA serves as the substrate for RT-PCR.
   D. RT-PCR generates a protein product, whereas PCR generates a DNA amplicon.
7. Immunoglobulin and T-cell receptor gene rearrangement studies can be used to: (Objective 5)
   A. distinguish B-cell leukemia from B-cell lymphoma
   B. determine whether a lymphoid clone is present in a tissue specimen
   C. prove that a tissue sample is benign
   D. detect Epstein-Barr virus in a tissue specimen
8. Which of the following is true about the molecular genetics of cancer? (Objective 3)
   A. Virtually all cancers are thought to harbor genetic defects.
   B. DNA testing can be helpful in making a cancer diagnosis.
   C. The genes responsible for tumor formation are called *oncogenes*.
   D. All of the above
9. Which of the following is true about the Southern blot procedure? (Objective 2)
   A. The patient's DNA is cut into fragments using proteinase enzymes.
   B. The electrophoresis step permits the probe to penetrate the gel.
   C. The probe is labeled so that it can hybridize to its complementary strand.
   D. Interpretation of results relies on visualization of the band pattern.
10. A technique that uses amplification of a patient sample target that is applied to a blot of WT and mutant oligonucleotides is called a(n): (Objectives 2,7)
    A. Southern blot
    B. qPCR
    C. direct sequencing
    D. ASO

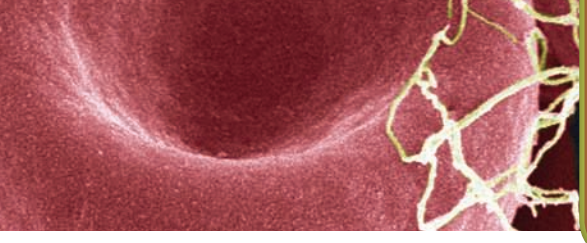
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Lefferts JA, Tsongalis GJ. Molecular assessment of human disease in the clinical laboratory. In: Coleman WB, Tsongalis GJ, eds. *Molecular Pathology: The Molecular Basis of Human Disease*, 1st ed. Burlington, MA: Elsevier; 2009:605–12.
2. Miler S, Seet H, Khan Y et al. Comparison of QIAGEN automated nucleic acid extraction methods for CMV quantitative PCR testing. *Am J Clin Pathol*. 2010;133:558–63.
3. Kessler HH, Muhlbaure G, Stelzl E et al. Fully automated nucleic acid extraction: MagNA pure LC. *Clin Chem*. 2001;47:1124–126.
4. Golub TR. Genomic approaches to hematology. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier Churchill Livingstone; 2013:16–26.
5. Wagner AJ, Berliner N, Benz EJ. Molecular and cellular basis of hematology. In: Hoffman R, Benz EJ Jr, Silberstein LE et al., eds. *Hematology: Basic Principles and Practice*, 6th ed. Philadelphia: Elsevier Churchill Livingstone; 2013:1–15.
6. Lam EP, Chan CM, Tsui NB et al. Clinical applications of molecular technologies in hematology. *J Med Diagn Methods*. 2013;2:1–6.
7. Gabert J, Beillard E, van der Velden VH et al. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia—a Europe Against Cancer program. *Leukemia*. 2003;17:2318–357.
8. Morley AA, Latham S, Brisco MJ et al. Sensitive and specific measurement of minimal residual disease in acute lymphoblastic leukemia. *J Mol Diagn*. 2009;11:201–10.
9. Farina L, Carniti C, Dodero A et al. Qualitative and quantitative polymerase chain reaction monitoring of minimal residual disease in relapsed chronic lymphocytic leukemia: early assessment can predict long-term outcome after reduced intensity allogeneic transplantation. *Haematologica*. 2009;94:654–62.
10. Langabeer SE, Gale RE, Harvey RC et al. Transcription-mediated amplification and hybridisation protection assay to determine BCR-ABL transcript levels in patients with chronic myeloid leukaemia. *Leukemia*. 2002;16:393–99.
11. Reyes AA, Carrera P, Cardillo E et al. Ligase chain reaction assay for human mutations: the sickle cell by LCR assay. *Clin Chem*. 1997;43:40-44.
12. Kim JH, Lebo RV, Cai SP et al. Prenatal diagnosis of unusual hemoglobinopathies. *Am J Med Genet*. 1994;50:15–20.
13. Wilding P. Miniaturization: DNA chips and devices. In: Bruns DE, Ashwood ER, Burtis CA, eds. *Fundamentals of Molecular Diagnostics*. St. Louis, MO: Saunders Elsevier; 2007: 80–90.
14. de Jonge HJ, Huls G, de Bont ES. Gene expression profiling in acute myeloid leukaemia. *Neth J Med*. 2011;69:167–76.
15. Futschick ME, Kemmner W, Schafer R et al. The human transcriptome: implications for the understanding of human disease. In: Coleman WB, Tsongalis GJ, eds. *Molecular Pathology: The Molecular Basis of Human Disease*. Burlington, MA: Elsevier. 2009: 123–46.
16. Liesveld JL, Lichtman MA. Chronic myelogenous leukemia and related disorders. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010; 1331–380.
17. D'Andrea AD. Susceptibility pathways in Fanconi's anemia and breast cancer. *N Engl J Med*. 2010;362:1909–919.
18. Alexander J, Kowdley KV. HFE-associated hereditary hemochromatosis. *Genet Med*. 2009;11:7–13.
19. Bhardwaj U, McCabe ERB. Multiplex-PCR assay for the deletions causing hereditary persistence of fetal hemoglobin. *Mol Diagn*. 2005;9:151–56.
20. Galanello R. Recent advances in the molecular understanding of non-transfusion-dependent thalassemia. *Blood Rev*. 2012;26 (suppl 1):S7–S11.
21. Natarjan K, Townes TM, Kutlar A. Disorders of hemoglobin structure: sickle cell anemia and related abnormalities. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010:1223–236.
22. Chaibunruang A, Fucharoen G, Jetsrisuparb A et al. Interactions of hemoglobin Lepore ($\delta\beta$ hybrid hemoglobin) with various hemoglobinopathies: a molecular and hematological characteristics and differential diagnosis. *Blood Cells Mol Dis*. 2010;44:140–45.
23. Prchal JT, Prchal JF. Polycyethemia vera. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010; 1223–236.
24. Paz-Priel I, Friedman A. C/EBPα dysregulation in AML and ALL. *Crit Rev Oncol*. 2011;16:93–102.
25. Chen W, Rassidakis GZ, Medeiros LJ. Nucleophosmin gene mutations in acute myeloid leukemia. *Arch Pathol Lab Med*. 2006;130:1687–692.
26. Ma W, Kantarjian H, Zhang X et al. Detection of nucleophosmin gene mutations in plasma from patients with acute myeloid leukemia: clinical significance and implications. *Cancer Biomark*. 2009;5:51–58.
27. Tan AY, Westerman DA, Carney DA et al. Detection of NPM1 exon 12 mutations and FLT3 -internal tandem duplications by high resolution melting analysis in normal karyotype acute myeloid leukemia. *J Hematol Oncol*. 2008;29;1:10.
28. Fuster O, Barragán E, Bolufer P et al. Rapid detection of KIT mutations in core-binding factor acute myeloid leukemia using high-resolution melting analysis. *J Mol Diagn*. 2009;11:458–63.
29. Kilpivaara O, Mukherjee S, Schram AM et al. A germline JAK2 SNP is associated with predisposition to the development of JAK2(V617F)-positive myeloproliferative neoplasms. *Nat Genet*. 2009;41:455–59.
30. Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia*. 2010;24:1128–138.
31. Hochhaus A, Schenk T, Erben P et al. Cause and management of therapy resistance. *Best Pract Res Clin Haematol*. 2009;22:367–79.
32. Spence JM, Rothberg PG, Wang N et al. Demonstration of array-based analysis for highly multiplexed PCR assays application to detection of IGH@-BCL2 translocations in FFPE follicular lymphoma specimens. *J Mol Diagn*. 2011;13:252–62.
33. Avvisati G. Newly diagnosed acute promyelocytic leukemia. *Mediterr J Hematol Infect Dis*. 2011;3:e2011064.
34. Rubnitz JE, Wichlan D, Devidas M et al. Prospective analysis of TEL gene rearrangements in childhood acute lymphoblastic leukemia: a Children's Oncology Group study. *J Clin Oncol*. 2008;26:2186–91.
35. Zhao XF, Reitz M, Chen QC et al. Pathogenesis of early leukemia and lymphoma. *Cancer Biomark*. 2011;9:341–74.
36. McMillin GA. Pharmacogenetics. In: Bruns DE, Ashwood ER, Burtis CA, eds. *Fundamentals of Molecular Diagnostics*. St Louis, MO: Saunders Elsevier; 2007;125–54.
37. Perrotta PL, Svensson AM. Molecular diagnostics in hemostatic disorders. *Clin Lab Med*. 2009;29:367–90.
38. Roberts HR, Key NS, Escobar MA. Hemophilia A and Hemophilia B. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010;2009–29.
39. Johnson JM, Ginsburg D. vonWillebrand disease. In: Prchal JT, Kaushansky K, Lichtman MA et al., eds. *Williams Hematology*, 8th ed. New York: McGraw-Hill; 2010;2069–88.



43 Quality Assessment in the Hematology Laboratory

CHERYL BURNS, MS
LUCIA MORE, MS

Objectives—Level I

At the end of this unit of study, the student should be able to:

1. Identify the components of a quality assessment program and match sources of error to each component (e.g., pre-examination component).
2. State the importance of a quality assessment program.
3. State the importance of documentation in a quality assessment program.
4. Describe the use of proficiency testing in the clinical laboratory, including required frequency.
5. Given data, use an appropriate method to determine the reference range for an analyte.
6. Define *universal precautions* and identify their source.
7. Demonstrate knowledge of Occupational Safety and Health Administration (OSHA) standards and their application in the clinical laboratory.
8. Given a material safety data sheet (MSDS), identify critical information.
9. Define *accuracy*, *precision*, *control material*, *mean*, and *standard deviation*.
10. Given the appropriate data, calculate the mean and standard deviation and create a quality control chart.
11. Interpret quality control results using established control charts.
12. Given test results, recognize complete blood count (CBC) data and/or histogram variations that indicate the presence of white blood cell (WBC), red blood cell (RBC), and platelet abnormalities.
13. Recognize complete blood count (CBC) data that indicate the presence of interfering substances such as lipemia, hemolysis, and icteria.
14. Identify coagulation test results that indicate a problem with sample integrity.

Objectives—Level II

At the end of this unit of study, the student should be able to:

1. Design methods of competency testing.
2. Apply and interpret statistics used in method evaluation.

(continued)

Chapter Outline

Objectives—Level II *(continued)*

3. Determine the components and interpret the results of a method evaluation study.
4. Determine and appraise a method's reportable range.
5. Interpret the Westgard rules and explain their use in evaluating quality control results.
6. Describe the use of moving averages to monitor red blood cell (RBC) data.
7. Assess the use of patient samples to monitor daily quality control in hematology.
8. Select the appropriate actions to take when abnormalities are detected in hematology or coagulation results.
9. Recommend procedures to correct for the presence of lipemia, hemolysis, and icteria.
10. Demonstrate the ability to use delta checks in a quality control program.

Key Terms

Analytical measurement range (AMR)
Analytical sensitivity
Analytical specificity
Analytical time
"Blinded" preanalyzed sample
Clinical Laboratory Improvement Amendments of 1988 (CLIA '88)
Clinical and Laboratory Standards Institute (CLSI)
Competency assessment
Correlation coefficient (*r*)
Current Procedure Terminology (CPT) code
Critical value
Delta check
Diagnosis related group (DRG) code
Health Insurance Portability and Accountability Act (HIPAA)
International Classification of Disease code, 9th or 10th revision (ICD-9, ICD-10)
Internal quality control program
Linearity
Material safety data sheet (MSDS)
Medical decision level
Outlier
Proficiency testing
Quality control (QC) limit
Random variation
Reference interval (RI)
Reportable range
Slope (*b*)
Split sample
Standard deviation (SD)
Systematic variation
Transcription error
Turnaround time (TAT)
Universal precaution
y-intercept (*a*)

Background Basics

The information in this chapter builds on the concepts learned in previous chapters. To maximize your learning experience, you should review these concepts before starting this unit of study:

Level I and Level II

- Describe the sample collection protocol for hematology and hemostasis procedures. (Chapters 36, 37)
- Summarize each of the routine hematology procedures, and give potential sources of error. (Chapter 37)
- Summarize the characteristics of an optimally stained peripheral blood smear, and give potential sources of error. (Chapters 10, 37)
- Summarize each of the screening coagulation tests used in the laboratory, and give potential sources of error. (Chapter 36)
- Describe the principles of cell counting used by the automated hematology instruments and principles of clot detection for the automated coagulation instruments. (Chapters 36, 39)

OVERVIEW

One of the most important responsibilities of a laboratory professional is to ensure the quality of test results. To accomplish this, laboratories must establish quality assessment and quality control programs. These programs consist of guidelines designed to ensure accurate testing and reporting of results. A protocol for reviewing patient results to determine whether results can be reported must be included. This chapter discusses components of these programs. Additional support materials are provided in this chapter's Companion Resources to facilitate understanding of certain concepts (e.g., application of Bull's testing algorithm to quality assessment of RBC parameters).

TEST CODING AND REIMBURSEMENT

Laboratory professionals are naturally concerned about the quality of laboratory test results; an additional consideration, of necessity, is ensuring that appropriate tests are being ordered and coded correctly so the laboratory can be reimbursed for the testing performed. In addition to the order for a particular test, the health care provider must supply information for purposes of reimbursement by Medicare and other third-party payors. This includes the World Health Organization's **International Classification of Disease, 9th revision (ICD-9)** or **10th revision (ICD-10)** codes that are used in most of the world. ICD-10 is scheduled to replace ICD-9 codes in the United States on October 1, 2015. The ICD coding system is internationally accepted as a method for coding medical diagnosis, conditions, and symptoms and the cause of death in all health care settings. One of its uses is to help evaluate medical necessity and determine reimbursement for tests and procedures.

Another coding system, the **diagnosis related group (DRG)**, was developed for Medicare as a part of the prospective payment system for inpatients. Reimbursement for care of hospitalized patients by almost all payers does not occur for each test or procedure but for the overall diagnosis, the DRG. There are about 500 DRGs. The DRG code is assigned when the patient is discharged and is severity adjusted (MS-DRG).

The laboratory uses the **Current Procedure Terminology (CPT) code** (Chapter 1) when billing for outpatient tests. The CPT code represents a number assigned to a given laboratory test (as well as medical, surgical, and other diagnostic services) by the American Medical Association's CPT Editorial Panel. For example, the ICD-9 code for a patient with pernicious anemia is ICD-9 281.0 and the CPT code for intrinsic factor antibodies is 86340. Third-party payors review a test's CPT code and determine the medical necessity for that specific test based on the ICD code for the patient's condition. Reimbursement is based on a predetermined rate within a geographic region for each particular test.

QUALITY ASSESSMENT

Laboratories must have an established quality assessment program as mandated by subpart K—Quality Systems for Nonwaived Testing of the **Clinical Laboratory Improvement Amendments of 1988 (CLIA '88)**.[1] A laboratory's quality assessment program should be designed to monitor all aspects related to testing patient samples[2,3] to ensure accurate testing and reporting of results from all samples submitted to the laboratory. Accrediting agencies such as the College of American Pathologists (CAP) and The Joint Commission monitor a program's comprehensiveness and quality. This chapter reviews the various aspects to consider in designing a comprehensive quality assessment program.

Basic Components

A common approach to developing a quality assessment program is to divide it into three components: (1) pre-examination (previously referred to as *preanalytical*), which deals with all aspects affecting the test outcome occurring before the testing procedure, (2) examination (previously referred to as *analytical*), which incorporates all aspects affecting the testing procedure itself, and (3) post-examination (previously referred to as *postanalytical*), which deals with aspects affecting the test outcome occurring after the testing procedure (Table 43-1 ★).

★ **TABLE 43-1** Comprehensive Quality Assessment Program

Pre-examination components	Patient test requisitions
	Patient preparation
	Sample collection protocol
	Sample transport protocol
	Sample processing protocol
	Sample acceptability and rejection criteria
	Sample storage
	Phlebotomy training
Examination components	Test method/procedure
	Reagents
	Internal quality control
	External quality control (proficiency testing)
	Instrument maintenance
	Linearity/reportable range determination
	Method evaluation (instrument comparison)
	Reference range determination or verification
	Personnel requirements
	Competency testing
	Continuing education
Post-examination components	Reviewing patient results
	Posting patient results
	Maintaining patient records
	Monitoring turnaround time
	Administering and reviewing customer satisfaction surveys
	Documenting maintenance

Pre-Examination Component

The testing process begins with the test order. Patient test requisitions should be designed to be user friendly and to provide adequate patient information. At a minimum, this information should include the patient's name and unique patient identifier, age, sex, diagnosis, test to be performed, and source of the sample.

The patient should receive appropriate information to prepare for the tests; for example, they must abstain from taking aspirin or aspirin-like medication before platelet function testing. The laboratory's sample collection procedure manual should provide this information in a format easily distributed to the patient and clear enough for that person to understand.

One of the most important factors affecting a test's outcome is sample collection[4–6] (Chapters 36, 37). As we often hear, "The test result is only as good as the quality of the sample." Many variables enter into the sample collection process that can affect the outcome (Table 43-2 ★). The sample collection procedure manual and a thorough educational program for the phlebotomist or the individual designated to perform the phlebotomy (i.e., nursing personnel) should address each potential error. In addition, staff should participate in periodic continuing education addressing sample collection problems and introducing new protocols.

When the sample has been collected, it must be properly labeled with patient's name, unique identification number, collection date and time, and phlebotomist's initials and then transported to the laboratory for processing and testing. If testing cannot be done immediately, the sample should be stored properly. For example, a sample for routine coagulation testing must be centrifuged and the plasma must be separated from the cells and stored at room temperature if testing is to be performed within 4 hours (Chapter 36). All information specific to the test to be performed should be included in the sample collection procedure manual.

Examination Component

The examination component addresses all factors involving the testing procedure itself. A test method procedure manual should be available in all laboratories. The procedures within this manual should follow the guidelines established by the **Clinical and Laboratory Standards Institute (CLSI)** and address each test, including its purpose, principle, sample requirements, reagents, quality control, step-by-step

★ **TABLE 43-2** Potential Sources of Errors in Sample Collection and Their Effect on Test Outcome

Source of Error	Effect on Test Outcome
Patient misidentification	Inaccurate test results
Hemolyzed sample	Dilutional effect on analytes, false increase of analytes, and decreased erythrocyte counts
Failure to properly mix by inversion collection tubes that contain anticoagulant	Clotted sample and falsely decreased cell counts or prolonged coagulation test results
Failure to fill collection tube properly	Under- or over-anticoagulated sample for coagulation testing with citrate tube
Failure to follow the order of the draw[a]	Cross-contamination with collection tube additives
Tourniquet application longer than 1 minute	Hemoconcentration of sample
Collection from an IV site	Dilutional effect on analytes
Time of draw	Analyte dependent (e.g., hemoglobin is highest in the morning)
Patient anxiety or crying	Analyte dependent (e.g., increases leukocyte count)

[a]*Order of the draw* refers to the suggested order in which different anticoagulant and nonanticoagulant collection tubes are filled from a single venipuncture. For example, a heparin collection tube should be filled before an EDTA collection tube to avoid contamination of the heparin collection tube with potassium EDTA (Chapter 37).

procedure, reporting method, reference interval(s), critical value(s) if appropriate, interpretation of results, potential sources of error, and references.[7,8]

Each laboratory should establish an **internal quality control program** to monitor its testing process to ensure accurate patient test results on a daily basis. Quality control is addressed in more detail in the "Quality Control" section in this chapter. In addition, each laboratory should establish an external quality control program to assess overall quality control, also known as **proficiency testing**, which monitors the specific laboratory's testing process by comparing it to that of peer laboratories.[9] This also is addressed in the "Proficiency Testing" section of this chapter.

Maintenance of analytical instruments (e.g., automated blood cell–counting instrument) must be performed as directed by the manufacturer, documented, and be easily accessible for troubleshooting quality control problems.

Individuals performing the testing procedures must meet the personnel requirements established by CLIA'88, which vary depending on the test procedure. Continuing education is also required to keep testing personnel aware of changes within the testing procedures and the practice of the profession.

Post-Examination Component

The post-examination component addresses factors that can affect the test result and its use in patient treatment.[10,11] Procedures should be established for reviewing patient results and identifying those results that require further attention, such as a **critical value** (test result that exceeds its reference interval to the extent that it indicates potential life-threatening condition requiring immediate attention by a physician) or results that do not match. For example, a sample should be retested if the hemoglobin (Hb) and hematocrit (Hct) do not match (e.g., Hb $\times$ 3 = Hct).

The laboratory should have a policy for reporting critical values. It should identify the laboratory professional (e.g., testing personnel or supervisor) who is responsible for notifying a health care provider of a critical value for a patient and the specific health care provider who should receive the test result (e.g., ordering physician). The method of communication can be phone notification or automated electronic messaging. The critical value record should include patient name, unique identification number, test result, date and time of notification, health care provider who received the test result, and laboratory professional who communicated the test result.

Automated instruments can be interfaced with the laboratory information system (LIS) to electronically transfer patient results. The LIS can also be interfaced with the hospital's or outpatient facility's computer system to add patient results directly on the patient's chart. Electronic transfer of results minimizes **transcription errors**. The records of patient test results should be maintained within the laboratory according to procedures established for encrypting, archiving, and retrieving patient test results.

The laboratory is a business enterprise. Therefore, customer (e.g., physician or patient) satisfaction and communication are important issues to be addressed in the quality assessment program. A quality assessment committee should be established to oversee the quality assessment program, establishing protocols and determining changes that need to be made and how to implement them. As an example, the committee establishes protocols to address customer complaints and other communication issues to minimize customer dissatisfaction. Surveys can be used to assess customer satisfaction and identify areas that need to be addressed. An important factor affecting satisfaction level is the **turnaround time (TAT)** for test results. Critical patient care decisions often depend on a laboratory test result. As a result, TAT problems represent a common complaint that the committee will investigate and determine the appropriate action to correct a specific TAT problem (e.g., delayed reporting of CBC results to the emergency department). Investigating TAT problems is more manageable with computerization (i.e., hospital information system [HIS] and LIS). The committee also reviews critical value records to identify problems with timely reporting of these values and suggests remedies to correct the problems.

In accordance with CLIA'88 and the **Health Insurance Portability and Accountability Act (HIPAA)**, each laboratory should establish certain measures to ensure the confidentiality of patient information in each component of the quality assessment program.[1,12]

For example, many facilities use unique identifiers rather than patient names or social security numbers to identify a sample and its test requisition. For the post-examination component, the laboratory should have a policy to ensure and document that the appropriate individual receives electronically transmitted test results.

Finally, documented records of all aspects of the quality assessment program should be maintained and retrievable upon request. These documents provide important information regarding the recognition of a problem, the process used to resolve it, and the change that occurred as a result of that process.

☑ CHECKPOINT 43-1

Explain the importance of each component of the quality assessment program to the goal it is intended to meet.

Proficiency Testing

Proficiency testing is a required component of a quality assessment program and represents an external quality control program that monitors the long-term accuracy of the different test systems (e.g., prothrombin time by the Beckman Coulter ACL TOP® instrument) through comparison to peer laboratories. Since the 1960s, many clinical laboratories have participated in proficiency testing surveys such as the CAP survey program. In addition, CLIA '88 mandated that all clinical laboratories performing nonwaived testing (testing methodologies not on the waived test list; Chapter 37) participate in a proficiency testing survey at least three times a year.[13] Failure to achieve an acceptable rating for any given analyte (e.g., prothrombin time) in two of three surveys can result in certain sanctions, such as delineation of a plan of corrective action for that test procedure or suspension of the certification to perform that test procedure, being placed on a laboratory.[14] The Centers for Medicare and Medicaid Services (CMS) issues the sanctions and penalties. To reinstate a test procedure, the laboratory must obtain an acceptable rating for that analyte in two consecutive proficiency testing surveys.

Laboratories contract with organizations such as CAP or the American Proficiency Institute to provide the proficiency testing service. A proficiency testing survey consists of unknown samples of whole blood or lypholized serum/plasma representing the full range of values that would be expected in patient samples. For microscopic procedures, the survey can include prepared slides and/or digital (photographic) images for evaluation and identification. These samples are sent to the laboratory at specified time intervals, usually three times per year. The laboratory should test proficiency samples as part of a typical patient sample run. Results are sent to the survey provider for statistical analysis. The survey provider determines the target value (TV) for each test result through comparison studies with peer laboratories and establishes the acceptable performance (AP) ranges based on the CLIA '88 tolerance limit (TL). For example, hemoglobin's tolerance limit is 7%. If the target value for hemoglobin sample 1 is 12.0 g/dL, the acceptable performance range is 11.2–12.8 g/dL ($AP = TV \pm TL \times TV$). The survey provider notifies the laboratory and CMS of its findings.

Each laboratory should have a comprehensive program to respond to an unsatisfactory result. The source of the problem that caused the unsatisfactory result can be identified by checking for changes in the test procedure or reagents, reviewing the instrument's maintenance log and previous quality control results, and identifying changes in testing personnel. When the problem has been identified, corrective action can be taken to solve it. The laboratory should maintain proficiency testing survey results and documentation of corrective action.

☑ CHECKPOINT 43-2

How does a laboratory that has lost its certification to perform protein C assays regain that certification?

Competency Testing

An additional required component of a quality assessment program is a **competency assessment** of all personnel performing nonwaived testing. This assessment takes place twice during the first year of employment and annually thereafter. CLIA '88 identified six elements of competency that must be evaluated (Table 43-3 ★) but did not clearly outline the exact mechanisms to use to evaluate competency.[15,16] Accrediting agencies such as The Joint Commission and CAP provide guidance to supervisors and laboratory directors in developing assessment tools through their standards and checklists (e.g., CAP's Laboratory General Checklist question GEN.55500). Competency assessment must include all six elements as appropriate for a given test procedure.

Direct observation checklists, random assignment of proficiency testing materials, or **"blinded" preanalyzed samples** can be used to evaluate elements of competency assessment (see Web Table 43-1 for an example of a direct observation checklist). If the test procedure involves microscopic examination for evaluation and identification, prepared slides or digital images can be used. Criteria for each assessment tool must be established to judge acceptable performance. In the case of a 100-cell leukocyte differential, an acceptable criterion might

★ **TABLE 43-3** Six Elements of Competency Assessment

1. Direct observation of routine patient test performance, including patient preparation if applicable, sample collection, handling, processing, and testing
2. Monitoring of the recording and reporting of test results
3. Review of intermediate test results or worksheets, quality control records, proficiency testing results, and preventive maintenance records
4. Direct observation of performance of instrument maintenance and function checks
5. Assessment of test performance by testing previously analyzed samples, internally blinded samples, or external proficiency testing samples
6. Assessment of problem-solving skills

be based on the 95% confidence limits of the expert (e.g., hematology supervisor or pathologist) results.[17] No single method evaluates all elements of competency nor is it appropriate for all test procedures. Additionally, educational materials (e.g., textbooks, selected journal articles, slide study sets, videotapes, computer-based instruction) should be available to assist laboratory professionals to improve their competency.

To help the laboratory meet this CLIA '88 requirement, CAP has developed its Competency Assessment Program.[18] This online program allows laboratory directors and supervisors to use established assessment tools and to develop laboratory-specific assessment tools for a given laboratory procedure. The program documents continuing education credits for each laboratory professional and maintains competency assessment records for the laboratory.

☑ CHECKPOINT 43-3

What is an appropriate method to assess a laboratory professional's competency in performing prothrombin time (PT) and activated partial thromboplastin time (APTT) using an automated coagulation instrument?

Method Evaluation/Instrument Comparison

Selection, evaluation, and implementation of a new methodology or instrument in the hematology/hemostasis laboratory should follow an established protocol. This section discusses several important components to be included.

Selection

Selecting a new methodology or instrument is a daunting task. In the ideal setting, a committee should be formed to do this. For a new instrument selection, committee membership can include the hematology/hemostasis supervisor, several laboratory professionals, LIS personnel, the quality assessment supervisor, a biomedical engineer, and the laboratory director.

This committee's first task is to determine the desirable characteristics of the new instrument.[19,20] A needs assessment survey could be used for this purpose; it should be completed by those individuals who will use the instrument and by those who might be affected by its use (Web Table 43-2). Desirable characteristics (Web Table 43-3) identified by this survey can then be used to solicit proposals from vendors (e.g., sales personnel for Beckman-Coulter, Siemens Medical, or Abbott Laboratories).

The selection committee's careful evaluation of the vendor's proposal packet narrows the selection process to several possible instruments. Committee members should also seek input from colleagues and the literature with regard to new instrumentation available and other laboratories' experiences with specific instrumentation. The in-house evaluation of each instrument is a crucial step in the selection process. At this time, all interested parties would have a hands-on opportunity to assess the actual performance of the instrument in a real-time laboratory. Thus, a more meaningful evaluation can be obtained with regard to whether the instrument meets the laboratory's needs. The more information the committee has on which to base its selection, the better the selection will be. Ultimately, the instrument selection is based on a particular laboratory's needs and the cost to meet them. For example, a full-service hematology laboratory requires an automated blood cell–counting instrument capable of performing a complete blood count (CBC), platelet count, five-part leukocyte differential, reticulocyte count, and immature reticulocyte fraction.

The process of selecting a new methodology or test system is similar. The selection committee must consider the cost per test, reagents, reagents' shelf-life and storage requirements, quality control program, test's **analytical sensitivity** (ability to detect small quantities of the analyte), **analytical specificity** (ability to determine only the analyte in question), and **linearity** (range of concentration over which the test method can be used), required instrumentation and equipment, **analytical time**, and sample types that can be analyzed (e.g., whole blood, serum, cerebrospinal fluid). Both testing personnel and potential clients should be consulted for their input during the selection process.

Analytical Reliability

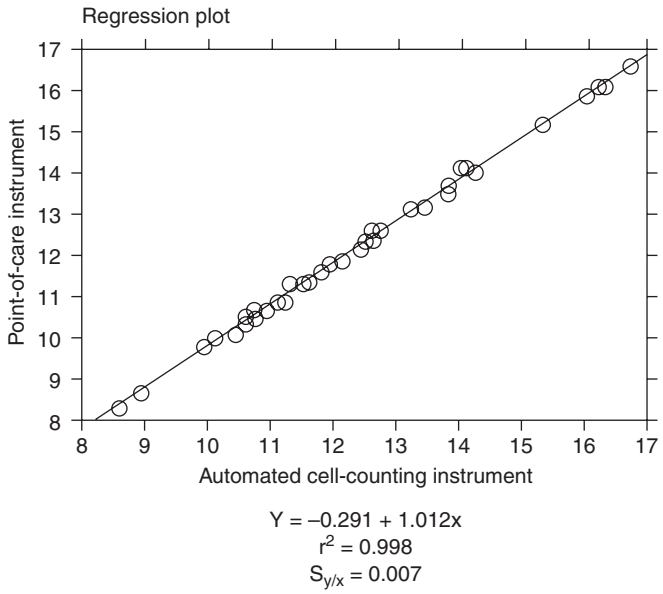
With the purchase of a new instrument or the introduction of a new methodology, the laboratory must verify the performance of the instrument and/or method through a series of performance studies. To verify an instrument's analytical reliability, the laboratory professional must evaluate the instrument with regard to **random variation** (variation resulting from chance) and **systematic variation** (variation within the instrument that alters results but is predictable). Precision studies are used to assess random variation and evaluate the reproducibility of the test method.[19,21] To check within run precision, the laboratory professional should choose at least two patient samples and analyze 10–20 aliquots of each sample in the same test run. These patient samples should have different concentration levels that correspond to an analyte's **medical decision levels** (concentration of an analyte indicating that medical interpretation is required for patient care). For example, to check within run precision for hemoglobin, three patient samples can be chosen: sample 1 Hb = 8.0 g/dL, sample 2 Hb = 12.0 g/dL, and sample 3 Hb = 19.0 g/dL. Each sample is separated into 10 aliquots each of which is analyzed. For each set of data, the mean, **standard deviation (SD)**, and coefficient of variation are calculated (Table 43-4 ★). Precision can be determined by applying a statistical test called the *F-test* or by comparing the calculated coefficient of variation (CV) to the manufacturer's CV. Within run precision is acceptable if the CV is less than or equal to the manufacturer's CV. If the CV is higher than the manufacturer's CV, the laboratory professional should check the data for **outliers** (data point that falls outside the expected range for all data). Any outlier should be discarded and the data re-evaluated. If the CV is still unacceptable, significant random variation exists within this method or reagent and/or testing personnel errors have affected the study.

Systematic variation is assessed through the methods comparison procedure, which allows comparison of patient results between the new method and a method that is known to be accurate (e.g., current method). **Split samples** (division of a single sample into two or more aliquots) are used. The CLSI recommends that at least 40 patient samples be tested over at least 5 working days.[21–23] To increase the reliability of this comparison, more patient samples should be used. The samples should be random so they represent the clinical range of

★ **TABLE 43-4** Within Run Precision Study for Hemoglobin Determination by Daman EXCELL-16

	Sample 1	Sample 2	Sample 3
1	7.9	12.0	19.2
2	8.1	12.3	19.4
3	8.0	12.2	19.4
4	8.1	12.2	19.4
5	8.1	12.2	19.3
6	8.0	12.3	19.4
7	8.0	12.3	19.4
8	8.1	12.4	19.5
9	8.1	12.4	19.3
10	8.1	12.3	19.6
Mean	8.1	12.3	19.4
SD	0.07	0.11	0.11
CV	0.86%	0.89%	0.57%
Manufacturer's CV	<1.0%	<1.0%	<1.0%

Three patient samples were chosen, and 10 aliquots of each sample were tested. The mean, standard deviation (SD), and coefficient of variation (CV) were determined for each patient sample. Comparison of each calculated CV to that of the manufacturer reveals acceptable precision for the hemoglobin procedure because the calculated CV is less than the manufacturer's CV. This procedure demonstrates the reproducibility of this hemoglobin determination. Results are reported in g/dL.



■ **FIGURE 43-1** Linear regression analysis for comparison of hemoglobin by automated cell-counting instrument (x-axis) and point-of-care instrument (y-axis). Data sets are given in Web Table 43-4. Interpretation of the linear regression analysis reveals a strong relationship, $r^2 = 0.998$, in the hemoglobin method between the automated cell-counting instrument and the point-of-care instrument. No proportional systematic error exists because the slope (1.012) is between 0.95 and 1.05. The y-intercept (−0.291) is slightly less than 0, which indicates a small degree of negative bias or constant systematic error. Most laboratories will consider this negligible. Random error also is not indicated because the standard error of the estimate (0.007) is nearly 0. Overall, this analysis demonstrates excellent comparison of methods.

samples. Ideally, they should represent different pathologic conditions as well (i.e., results outside the reference interval). With the samples identified, each is split for analysis by each method. The samples are tested in duplicate for each method, and replicate testing should be done during the same test run. Analysis of a given sample should be completed within 2 hours on both methods and the results documented. Before statistical analysis can be performed, the results should be examined to determine whether any outliers exist, the results are linear, and range of results is adequate. Detection of outliers and determination of linearity can be made by plotting the new method results (Y) versus the current method (X). The coefficient of determination (r^2) is used to determine whether adequate range exists. If the results are linear and r^2 is greater than or equal to 0.95, simple linear regression analysis can be used. Linear regression analysis allows for the determination of the **y-intercept (a)**, **slope (b)**, standard error of the estimate ($s_{y/x}$), **correlation coefficient (*r*)**, and coefficient of determination (r^2) (Figure 43-1 ■). The general formula for the linear regression line is $y = a + bx$, where y is the predicted mean value of y for a given x value. The coefficient of determination evaluates the strength of the relationship between the two methods. For example, an r^2 value of 0.90 for a comparison between current and new methods means that 90% of the variability in the new method is directly predictable from the variability in the current method. Therefore, a strong relationship exists between the two methods.

The paired *t* test can be included as a statistical tool; it compares the mean of the differences of test results for the two methods and determines whether a statistically significant difference exists between the current method and the new method (see Web Table 43-4 for an example of a paired *t* test). The calculated *t*-value for the two sets of results is compared to the critical *t*-value from a statistical table. If the calculated *t*-value is less than the critical *t*-value, no significant difference exists between the two methods.

Linear regression analysis is also used to detect systematic (constant or proportional) and random errors. Constant systematic errors are identified by a change in the y-intercept. A y-intercept with a value other than 0 ($y > 0$ or $y < 0$) indicates that a constant difference exists between the new method and the current method regardless of the analyte's concentration. The observation of a constant systematic error usually indicates a calibration problem. Proportional systematic errors are identified by changes in the slope. A change in the slope represents a difference between the new method and the current method that is proportional to the analyte's concentration. That is, the higher the concentration, the greater the difference is between the two methods. If there is no difference between the current method and the new method, the slope is $1.00 \pm 5\%$. A proportional systematic error is most frequently associated with erroneous calibration. Random error can be detected by an increasing standard error of the estimate. Increased dispersion of results about the regression line causes an increased standard error of the estimate. No standard criteria exist for the interpretation of an acceptable standard error of the estimate. Thus, the result should be evaluated in conjunction with the results of the precision studies.

☑ CHECKPOINT 43-4

Linear regression analysis was performed on results from a method comparison of the prothrombin time between automated coagulation instrument A and automated coagulation instrument B (Web Figure 43-1). The following results were obtained:

γ-intercept = 0.8157

Slope = 0.9982

Standard error of the estimate = 0.0807

What conclusions can be drawn from these results?

Linearity and Reportable Range Determinations

The manufacturer determines an instrument's linearity or **analytical measurement range (AMR)**. For hematology instruments, linearity is determined for each directly measured parameter (Table 43-5 ★). Verification of the instrument's **reportable range** (i.e., analytical measurement range) must be included in the method evaluation procedure for a new hematology or coagulation instrument or as one of the installation procedures for that new instrument.[24] To verify the reportable range, the laboratory professional should select linearity check samples (i.e., linearity check materials and/or fresh human whole blood samples) that span the instrument's established linear range for each directly measured parameter. These linearity check samples are then analyzed multiple times to minimize the effect of imprecision on the linearity study's results.

After the data are examined for possible imprecision, they are plotted (known result versus measured result) to allow visual assessment of the linearity of these results. The degree of nonlinearity at each level is also determined. This degree of nonlinearity or nonlinear error is compared to the predetermined goal for nonlinear error established by each laboratory following the guidelines outlined in the CLSI *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*.[25] Alternatively, the manufacturer of the linearity check materials can provide data analysis for a laboratory's linearity study. The laboratory follows the instructions supplied by the manufacturer to perform the linearity study and submits a spreadsheet with the data. The manufacturer then evaluates the data and returns a printed document that details the linearity study results. If they fall within the laboratory's acceptable range for nonlinear error, the reportable range is verified. If the results do not fall within the acceptable range, the process should be repeated using more linearity check samples in the affected part of the range. If the results do not verify the instrument's reportable range, the laboratory should modify the reportable range to reflect the instrument's performance characteristics in its current setting.

In addition, The Joint Commission requires that calibration verification be performed on hematology and coagulation instruments every six months if the instrument has a calibration process. The calibration verification essentially determines the linear range as just described and requires a minimum of three levels of linearity check samples that span the analytical measurement range or reportable range for each parameter.

With the verification of the reportable range, each laboratory should establish its protocol for handling results that exceed the reportable range, either above or below. For example, samples with results that are above the reportable range are diluted and retested, and the subsequent result is multiplied by the dilution factor to obtain the accurate result. Hematology results below the reportable range require retesting and review of the peripheral blood smear before the result is reported as being less than the lower limit of the reportable range.

Reference Interval Determination

Hematology and coagulation **reference intervals (RIs)** are available in various recognized hematology textbooks and the manufacturer's hematology/coagulation instrument and reagent manuals. To use these RIs, a laboratory must verify that the RIs are appropriate for its patient population as required by CLIA '88.[24] Diversity of instrumentation, choice of reagents, and patient population served by the laboratory influence RIs. The laboratory can choose to validate a manufacturer's RIs or establish an RI. Validating an RI is less time consuming and more cost effective. The recommended procedure for validating an RI is described in Web Table 43-5.[26] Once validated,

★ **TABLE 43-5** Display Range and Analytical Measurement Range (AMR) for Abbott Cell-Dyn Emerald Instrument

Parameter	Units[a]	Display Range	AMR
WBC	K/mcL (μL)	0–100	0.4–96.1
RBC	M/mcL (μL)	0–8	0.22–7.61
Hb	g/dL	0–25	3.3–24.6
Hct	%	0–80	5.3–75.6
MCV	fL	0–150	48.8–115
Platelets	K/mcL (μL)	0–1500	9–1375

WBC = white blood cell; RBC = red blood cell; Hb = hemoglobin; Hct = hematocrit; MCV = mean corpuscular volume; K = 10^3; M = 10^6

[a]Results are expressed in Standard (US) units.

Courtesy of Abbott Laboratories, Abbott Park, IL.

the RIs can be used as representative for the laboratory and its patient population. If the RIs are not validated, the laboratory can choose to establish their own RIs, as described below.

Establishing an RI is an arduous task. It involves careful planning to define the criteria for subject selection, the data acquisition process, and the data analysis. In the ideal situation, RIs should be established based on the patient population's age and sex stratification. For reliable estimates of an RI, a minimum of 120 individuals in each age and sex category should be tested.[26] One method of categorizing age groups is by decade of life. However, simple mathematical calculation would show an overwhelming number of individuals needed for such a process. Winsten suggests a mechanism to decrease the number of individuals needed by dividing the patient population into four age categories[27] (Table 43-6 ★). All subjects should provide brief histories to determine their acceptability for the study and be given the appropriate instructions to prepare for the blood draw. In addition, each subject must sign an institutional review board (IRB) approved consent form. Ideally, 5–10 subjects should be tested per day to minimize possible random introduction of a shift in the RI due to instrument or reagent differences.

When all data have been acquired, they must be analyzed. Computer-based spreadsheets facilitate data analysis. CLSI recommends the use of percentile analysis, a nonparametric method,[26] which is appropriate because the analysis does not make any specific assumptions regarding the distribution of data points (e.g., Gaussian or non-Gaussian distribution). Using percentile analysis, the upper and lower limits of the RI depend on the ranks of reference data arranged in order of increasing values. The lower limit identifies the estimated 2.5th percentile, and the upper limit identifies the estimated 97.5th percentile, thus defining the 95% RI (see Web Table 43-6 for an example of percentile analysis to determine an RI).

☑ CHECKPOINT 43-5

Define the term *reference interval*.

Safety

The laboratory environment includes biohazards, chemical hazards, and physical hazards. All laboratory employees must know the requirements for performing their jobs in a manner that protects them and their coworkers from these hazards. Several governmental agencies have established guidelines and standards for ensuring the safety of the laboratory professional.

★ **TABLE 43-6** Winsten's Recommended Age Categories for Reference Interval Determination

1. Newborns
2. Prepubertal individuals
3. Adult (includes postpubertal and premenopausal)
4. Older adults (includes males >60 years and postmenopausal females)

Universal Precautions

The Centers for Disease Control and Prevention (CDC) introduced **universal precautions** in 1982.[28] With the discovery of the human immunodeficiency virus (HIV) and its potential transmission through exposure to infected blood or other body fluids, the CDC recognized the need for preventative guidelines to minimize potential exposure of health care workers to this virus. Universal precautions state that health care workers should consider all body fluids as potentially infectious. Therefore, health care workers must use the appropriate personal protective equipment (PPE) when handling body fluids to minimize the risk of exposure to biohazardous agents such as HIV, hepatitis B, and other blood-borne pathogens (Chapter 37).

The CDC has updated the original guidelines and continues to do so. Current guidelines recommend that all health care workers receive the hepatitis B vaccine. See Web Table 43-7 for selected recommendations from the CDC and the Occupational Health and Safety Administration (OSHA) designed to minimize the potential exposure to and transmission of blood-borne pathogens to laboratory professionals.[29,30] Within a health care facility such as a hospital or medical center, the term *standard precautions* is used to define the facility's policies regarding universal precautions and infection control.

Occupational Health and Safety Administration (OSHA) Standards

OSHA regulates many aspects of the clinical laboratory to ensure a safe work environment. Each clinical laboratory must meet OSHA's standards for chemical, physical, and fire safety. For biologic safety, OSHA implemented the Blood-borne Pathogen Standards in 1992. All OSHA standards require educating and training laboratory employees, implementing an exposure control plan, and using a record-keeping mechanism. OSHA's website (www.osha.gov) is a source of additional information and details regarding these standards.

Material Safety Data Sheets

The **material safety data sheet (MSDS)** provides safety information for laboratory professionals who use hazardous materials. Web Figure 43-2 is an example of one. It includes pertinent safety information regarding the following for a chemical: proper storage and disposal, precautions that should be taken in handling it, potential health hazards associated with exposure to it, and whether the chemical is a fire or explosive hazard. Under the Hazard Communication Standard, or "Right to Know Law," laboratory professionals must receive training regarding the hazardous chemicals they work with.[31] This training should include the potential health risks associated with the chemicals, interpretation of MSDS and chemical labels, and review of the laboratory's hazard communication program. MSDSs must be available at all times to the laboratory professionals.

☑ CHECKPOINT 43-6

If a spill occurred when handling the CELL-DYN CN-free Sapphire hemoglobin reagent, what is the appropriate procedure to clean it up? Refer to Web Figure 43-2.

QUALITY CONTROL

The clinical laboratory's quality control program is a critical component of the quality assessment program and monitors the testing process to ensure that reliable test results are obtained for the patient samples, to detect potential problems within the testing system, and to allow correction of the problem before patient results are affected.

Control Materials

Control materials are assayed samples with predetermined test results. The manufacturer assigns a lot number to each batch of control material. Within a given lot number, the assayed characteristics of the control are the same. Most hematology procedures use stabilized cell suspensions that closely match the characteristics of human whole blood. The stability of cell suspensions is limited. For most commercially available cell suspensions, the time from a given lot number's start date to its expiration date is 4 months. For coagulation procedures (e.g., PT and APTT), lyophilized control materials are used. When reconstituted, the lyophilized control has behavioral characteristics similar to platelet-poor citrated plasma. A given lot number for these lyophilized controls is ordered in sufficient quantities to meet the laboratory's testing needs for 1 year. Because a new control limit must be determined for each new lot number of control, this is advantageous decreasing the laboratory's time spent on determining control limits and providing a continuous monitor of the testing process over reagents and laboratory personnel changes.

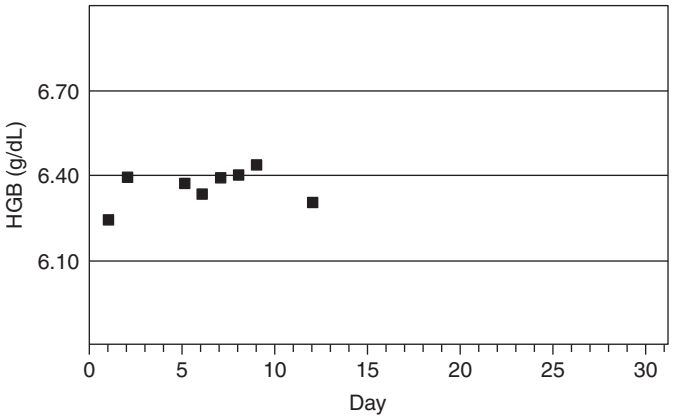
Establishing Quality Control (QC) Limits

Quality control (QC) limits must be established for each control material before its use within the quality control program. Standard protocol for determining quality control limits calls for testing a new control material to collect a minimum of 20 data points (control measurements) over 10 working days while monitoring the integrity of the testing process with the current control material.[32] Initial control limits are determined using this data. As more data points are collected, however, the limits should be recalculated using all data points to establish truly reliable control limits. The statistics used to establish the QC limits are the mean (x) and SD. Using the mean and SD, the control limits $\pm$1s, $\pm$2s, and $\pm$3s, are calculated.

The mean and control limits are then used to establish the QC chart (also known as a *Levey-Jennings chart*) (Figure 43-2 ■). The QC chart is used to plot the control material results over time to provide a graphical display of the distribution of control results over a given period, usually 1 month.

☑ CHECKPOINT 43-7

To establish the control limits for a new lot number of level 1 PT coagulation control, the following data points were collected (results are in seconds): 11.8, 11.6, 12.1, 12.0, 12.3, 12.6, 11.9, 12.2, 12.0, 11.5, 12.7, 12.1, 11.2, 12.3, 12.9, 13.0, 12.3, 11.9, 12.4, and 12.5. What are the $\pm$2s control limits?



■ **FIGURE 43-2** Quality control chart for level 1 hemoglobin control depicting control results for days 1-12. The $\pm$2 SD limits are 6.10–6.70 g/dL, with a mean of 6.40 g/dL. The quality control results would be interpreted as being in control.

Interpreting Quality Control Charts

Statistically, 95% of the control results should fall within $\pm$2 SD, and 99% should fall within $\pm$3 SD. Careful and continual evaluation of the QC charts alerts the laboratory professional to potential problems in the testing process before a serious breakdown in the test's integrity occurs. By using the Westgard multirule approach, the problem can be identified and corrected.

Westgard Rules

James O. Westgard, Ph.D., and colleagues developed the Westgard rules to evaluate control results when two or more levels of control material are used.[33] See Table 43-7 ★ for lists and definitions of the most commonly used Westgard rules.

Evaluation of Quality Control Charts Using the Westgard Multirule Approach

Each laboratory creates its multirule protocol for a given instrument by selecting a combination of Westgard rules. The selection depends on the acceptable level of false rejection (rejection of a control run that is not truly out of control) and error detection (rejection of a control run when a true error is detected) and the number of control levels run on that instrument.[34] The goal of the multirule protocol is to minimize the chance of false rejection of a control run and maximize the ability to detect true error. For example, the multirule protocol for an instrument using two control levels might be $1_{2s}/1_{3s}/2_{2s}/R_{4s}/4_{1s}/10_{x}$. Westgard uses *S* as an alternate abbreviation for SD. The 1_{2s} rule is used as a warning to indicate the possibility that a rule has been violated. If a 1_{2s} warning is observed for one of the control results from the current test run, the laboratory professional should evaluate the QC charts and consider previous control results to determine whether a violation has occurred (Figure 43-3 ■).

Depending on the violation, an identified problem can be classified as a random or systematic error. A random error occurs by chance and can result from missampling or misidentifying the control. This type of error can be identified and corrected by carefully repeating the control. Systematic errors indicate a problem within the testing

★ **TABLE 43-7** Westgard Rules

Westgard Rule	Definition	Type of Error
1_{2s}	One control result exceeds a 2s limit. This is considered a warning of a potential out-of-control problem.	
2_{2s} for across runs	Two consecutive control results exceed the same 2s limit. Run should be rejected and out-of-control problem investigated.	Systematic
4_{1s} for across runs	Four consecutive control results exceed the same 1s limit. Run should be rejected and out-of-control problem investigated.	Systematic
10_x for across runs	Ten consecutive control results exceed the mean in the same direction (e.g., results are above the mean). Run should be rejected and out-of-control problem investigated.	Systematic
4_{1s} for across runs and between control levels	Two consecutive control results for two control levels exceed the 1s limit in the same direction across the last two control runs. Run should be rejected and out-of-control problem investigated.	Systematic
10_x for across runs and between control levels	Five consecutive control results for two control levels exceed the mean in the same direction across the last five control runs. Run should be rejected and out-of-control problem investigated.	Systematic
2_{2s} for within run	Two consecutive control results for two control levels exceed the 2s limit in the same direction for the current control run. Run should be rejected and out-of-control problem investigated.	Systematic
1_{3s} for within run	One control result exceeds a 3s limit. Run should be rejected and out-of-control problem investigated.	Random
R_{4s} for within run	One control result exceeds +2s limit, and the other control result exceeds −2s limit when using two control levels. Run should be rejected and out-of-control problem investigated.	Random

These rules can be applied "across runs" for two or more runs of a single control or both controls or "within run" for a single control or both controls.

system, which can result from poor calibration, a change in reagent or an expired reagent, expired or improperly stored control, or deteriorating light source. Review of the daily and periodic maintenance logs for the instrument can help identify the problem. Once the problem has been identified, the correct solution can be implemented.

☑ CHECKPOINT 43-8

After performing daily quality control on the automated hematology instrument, the laboratory professional observes a 4_{1s} violation for the hemoglobin parameter. What type of error is indicated?

Bull's Testing Algorithm (Moving Averages)

Moving averages (continuous statistical analysis on consecutive patient erythrocyte indices by an automated cell-counting instrument) is a method of using the erythrocyte indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) to monitor the instrument's performance in determining the erythrocyte parameters (e.g., erythrocyte count or hemoglobin).[35] Bull's testing algorithm (X-B analysis) represents a calculation of the moving averages for erythrocyte indices of the patient population. It is based on the premise that the erythrocyte indices within a patient population are stable. Therefore, moving averages can be used to monitor the precision and accuracy of the instrument's performance.

To establish the acceptable ranges for the moving averages, MCV, MCH, and MCHC, the erythrocyte indices on 500 consecutive patient samples are determined and the mean for each index is calculated. The acceptable range for each index is ±3% of the mean; however, each laboratory should determine its acceptable range.[36] These ranges are entered into the instrument's computer. The majority of hematology instruments calculate the moving average from each group of 20 patient samples and determine whether those moving averages fall within the acceptable range (Web Figure 43-3). The instrument alerts the laboratory professional if the moving average exceeds the acceptable range. If a moving average is unacceptable, the laboratory professional should identify the previous 20 samples because this method of monitoring moving averages of an erythrocyte index is sensitive to the patient population. If the previous 20 samples were patients from the renal dialysis clinic or oncology clinic, the change in moving averages could result from the patient population, not an instrument problem. A true alert would indicate an instrumentation problem affecting one or more of the erythrocyte parameters: erythrocyte count, hemoglobin, or hematocrit (Chapters 37, 39).

Monitoring Quality Control with Patient Samples

Patient samples can be retained and used in conjunction with purchased control materials to monitor a hematology instrument's reproducibility over a 24-hour period as a result of the stability of cell counts in an ethylenediaminetetracetic acid (EDTA)–anticoagulated sample. For example, four or five patient samples are selected from the morning test run, each sample is analyzed to determine its mean, and the samples are separated into two sets. The first set of separated samples that is tested every 4 hours is used to monitor the precision of the instrument during a 24-hour period. Using the SD and CV for these data, the instrument's precision is evaluated to determine its acceptability. The second set of separated samples that is refrigerated for 24 hours monitors precision from day to day. After 24 hours, the second set is analyzed to determine each sample's 24-hour mean. The original mean is compared to the 24-hour mean to determine acceptability. Each laboratory should establish its own limits for acceptability.

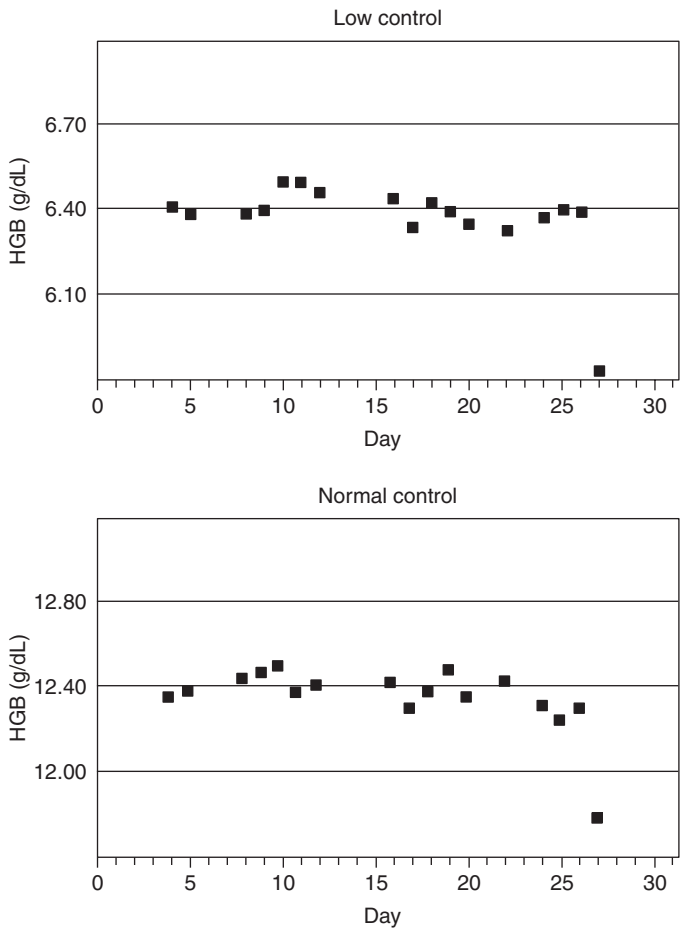


FIGURE 43-3 Quality control charts for hemoglobin controls depicting violation of 2_{2s} rule. Inspection of the current run, day 27, reveals that both control materials exceed the −2s limit, indicating a violation of the 2_{2s} rule within a run. This run should be rejected and the problem resolved before patient samples can be run. Because the 2_{2s} violation indicates a systematic error, the laboratory professional should investigate the possibility of a reagent, control, or instrument problem.

Another potential use of retained patient samples is as quality control materials for the laboratory's secondary hematology instrument. Patient samples analyzed on the primary hematology instrument, which has been determined to be in control by purchased control materials are analyzed on the secondary instrument. The results are compared to the primary instrument's results and, if comparable, the secondary instrument is considered in control during the same time interval.

REVIEW OF PATIENT RESULTS

Although automated verification or autoverification of patient results can be used in the hematology/hemostasis laboratory, the underlying criteria for the process come from the laboratory's protocol for review patient results[37] (Web Table 43-8). Automated verification allows a faster turnaround time for patient results. The laboratory professional is responsible for addressing those patient results that require special attention. This section describes review protocols and certain corrective actions for abnormal results in hematology and hemostasis.

Hematology

Laboratory professionals use the review protocol to examine patient data obtained from an automated cell-counting instrument and determine whether the CBC results can be reported or further action is required.

Detection of Abnormal Test Results

The initial identification of potential abnormalities in the CBC results is accomplished by the instrument's computer system, which is programmed to evaluate the numerical data, histograms, and scattergrams and to generate suspect flags and user-defined flags (e.g., definitive flags) (Chapter 39). The numerical data, histograms, scattergrams, and alert flags provide the laboratory professional information that can indicate the presence of interfering substances, abnormal cell morphology, or abnormal cells. See Table 43-8 ★ for common abnormal results or alert flags and corrective actions taken as a result.[38]

☑ CHECKPOINT 43-9

In reviewing a patient's CBC results, the laboratory professional notes an MCHC of 37 g/dL. What corrective action should be taken?

On occasion, CBC results that are spurious (e.g., results that first seem to be accurate but on review are invalid) are obtained. These results might not be grossly abnormal or flagged by the instrument's computer, but the results do fall significantly outside the reference interval. The laboratory professional should be alert for this possibility because spurious results can indicate a problem with the sample itself. Before these CBC results can be reported, the sample should be examined for potential sources of error (e.g., clots, lipemia, and agglutination). Table 43-9 ★ reviews the spurious hematology results that can be encountered in the clinical laboratory including the underlying problem that led to the erroneous parameter and the possible causes.[39–44]

Use of Delta Checks

Delta checks rely on consecutive testing of a particular patient. Comparison of current hematology results to the most recently reported previous result for a given patient allows the detection of certain random errors.[45] This method of error detection is termed the delta check and has been one of the greatest benefits of the LIS (Web Figure 43-4).

Limits can be defined to determine the allowable difference among consecutive results of a specific test (e.g., hemoglobin) over an established time interval. The limits define when the LIS will flag a result. The delta check difference can be calculated either as a difference in the absolute value or as a percentage of the difference. Regardless of the method used, the delta limit should be set so that true changes in test results are not flagged as delta check failures. If the time limit between comparisons and the maximum allowable

★ TABLE 43-8 Common Abnormal Results or Alert Flags and Their Corrective Actions

Abnormal Result/Alert Flag	Confirmation/Corrective Action	Rationale
Hemoglobin <7.0 g/dL	Confirm on alternate instrument.	A hemoglobin <7.0 g/dL is a critical value and should be confirmed before it is reported.
Hemoglobin × 3 does not equal hematocrit ±3	Confirm on alternate instrument or perform manual hematocrit.	This rule applies to normal individuals only and is used to identify common interfering substances in hematocrit determination (MCV) or hemoglobin determination (see MCHC).
MCHC > 36 g/dL	1. Perform manual hematocrit. 2. Check for presence of cold agglutinins, lipemia, or icteria. 3. Incubate blood sample at 37°C for 15 minutes, mix and analyze whether cold agglutinins are present.	Poor correlation between hemoglobin and hematocrit and an MCHC > 36 g/dL is associated with cold agglutinins, lipemia, and icteria. Cold agglutinins cause erythrocyte clumping and result in falsely elevated MCV, falsely decreased RBCs, falsely decreased hematocrit, and MCHC > 36 g/dL. Lipemia causes a falsely elevated hemoglobin because of the increased turbidity of sample and MCHC > 36 g/dL.
MCHC > 36 g/dL, no correction after warming to 37°C	Examine peripheral blood smear for presence of spherocytes.	Spherocytes have a decreased surface area-to-volume ratio and typically have an MCHC > 36 g/dL. After ruling out the possibility of cold agglutinins, the presence of spherocytes should be suspected.
Platelet count <50 × 10^3/mcL	1. Perform platelet estimate on peripheral blood smear and examine feather edge for platelet clumps. 2. Examine for grapelike cluster clumps of platelets associated with EDTA-dependent agglutination. If present, recollect using sodium citrate anticoagulant and multiply result by 1.1 (dilution factor). 3. Examine for platelet satellitism.	Presence of platelet clumps or platelet satellitism results in falsely decreased platelet counts. Platelet satellitism and platelet clumps can be EDTA dependent. Therefore, use of sodium citrate as the anticoagulant circumvents the mechanism of platelet clumping and platelet satellitism and allows determination of the platelet count.
Platelet count >900 × 10^3/mcL	Perform platelet estimate on peripheral blood smear. Platelet estimate should agree within ±25% of the platelet count. Examine for presence of erythrocyte fragments.	Poor correlation between platelet estimate and platelet count could be caused by the presence of erythrocyte fragments that are similar in size and volume to platelets. Erythrocyte fragments can cause a falsely elevated platelet count.
WBC <2.0 × 10^3/mcL or >30.0 × 10^3/mcL	Perform leukocyte estimate on peripheral blood smear. Leukocyte estimate should agree within ±25% of the leukocyte count.	Poor correlation between leukocyte estimate and leukocyte count can be caused by instrument errors or sample collection error.
MCV <70 fL, >110 fL (adult), >120 fL pediatrics	Review peripheral blood smear for microcytosis or macrocytosis.	Clinically significant changes in MCV are associated with nutritional deficiencies and thalassemias.
Automated differential flagged	Perform manual differential.	Confirm presence of abnormal cells or abnormal erythrocyte morphology.
Automated differential vote out	Perform manual differential.	Because of the presence of abnormal cells, the instrument is unable to properly classify the cells and determine the automated differential. The leukocyte differential must be obtained by performing a manual differential.
Microcytic/fragmented RBC flags	1. Review erythrocyte morphology on peripheral blood smear. 2. Perform platelet estimate on peripheral blood smear.	Microcytic erythrocytes or erythrocyte fragments are similar in size and volume to platelets. Therefore, the presence of microcytic cells/fragments can result in a falsely elevated platelet count. Poor correlation between the platelet estimate and platelet count is associated with this observation.
WBC, RBC, or platelets > upper limit of established reportable range (or + + + + +)	Perform a ½ or ⅓ dilution of the sample with diluent. Run the sample and multiply the result by the dilution factor. Report the elevated parameter from the diluted sample only.	Cell counts that exceed the upper limit of their reportable range require a dilution of that sample to lower the number of cells present and bring the diluted cell count into the instrument's reportable range. The actual cell count is then obtained by multiplying the diluted sample's cell count by the dilution factor.

WBC = white blood cell; RBC = red blood cell; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration

★ TABLE 43-9 Spurious Hematology Results, Underlying Problems, and Possible Causes [a, b, c]

Erroneous Parameter	Underlying Problem	Possible Cause
Pseudoleukocytosis	Platelet irregularities	Platelet clumps, very large platelets, EDTA-induced platelet aggregation
	Unexpected cell type	Nucleated erythrocytes (if not directly enumerated by instrument), micromegakaryocytes, megakaryocyte fragments
	Unlysed erythrocytes	Presence of abnormal hemoglobins such as HbC, patients receiving chemotherapy, cold agglutinins
	Abnormal precipitates	Cryoglobulinemia, cryofibrinogenemia, fibrin strands
	Intraerythrocytic parasites	Malarial parasites
	Carryover between samples in leukocyte dilution chamber	Extreme leukocytosis in previous sample
Pseudoleukopenia	Cell lysis	Storage artifact, leukemia (especially CLL), uremia, immunosuppression
	Aggregates counted as single cells	Presence of segmented neutrophil aggregates induced by certain anti-coagulants such as EDTA
	Sample collection issue	Clotted sample
Pseudoincreased hemoglobin	Turbidity	Elevated leukocyte count, hyperlipemia, paraproteinemia, cryoglobuli-nemia, cryofibrinogenemia, abnormal hemoglobin (e.g., HbS and HbC)
	Interference at 540 nm	Carboxyhemoglobinemia
Pseudodecreased hemoglobin	Interference at 540 nm	Sulfhemoglobinemia
	Unlysed erythrocytes	Presence of abnormal hemoglobins (e.g., HbC), patients receiving chemotherapy
	Inappropriate dilution	Overfilled collection tubes
Pseudoincreased RBC count	Other cells counted	Elevated leukocyte count, elevated numbers of very large platelets
	Abnormal precipitates	Cryoglobulinemia, cryofibrinogenemia
Pseudodecreased RBC count	Aggregates counted as single cells	Cold agglutinins, warm agglutinins
	Very small erythrocytes that fall below lower threshold for RBC count	Erythrocyte fragments, microcytes (MCV $<$50 fL)
	Sample collection issue	In vitro hemolysis, clotted sample
Pseudoincreased MCV	Other cells sized as RBCs	Elevated leukocyte count
	Aggregates sized as single cells	Cold agglutinins, warm agglutinins
	Osmotic swelling	Hyperglycemia, hypernatremia, K2 EDTA in excess, storage at room temperature
Pseudodecreased MCV	Instrument-related artifact	Hypochromic erythrocytes
	Other cells/particles sized as RBCs	Very large platelets, cryoglobulinemia, cryofibrinogenemia
	Osmotic shrinking	Hypo-osmolar states such as hyponatremia
Pseudoincreased hematocrit, automated	Artificial increase in MCV	Elevated leukocyte count ($>$50,000 mcL [μL]), hyperglycemia, hypernatremia
	Artificial increase in erythrocyte count	Cryoglobulinemia, cryofibrinogenemia, elevated numbers of very large platelets
Pseudodecreased hematocrit, automated	Artificial reduction in MCV	Hypo-osmolar states
	Artificial reduction in erythrocyte count	Cold agglutinins, warm agglutinins, erythrocyte fragments, microcytes, in vitro hemolysis, clotted sample
Pseudoincreased hematocrit, manual	Increased plasma trapping	Polycythemia, microcytosis, presence of sickle cells, spherocytosis
		Centrifuge force too low, decreased centrifugation
		Burn victim
	Decreased erythrocyte flexibility	Prolonged storage
Pseudodecreased hematocrit, manual	Decreased plasma trapping	Prolonged centrifugation, increased centrifugal force
	Sample collection issue	In vitro hemolysis
	Technique-related issue	Incomplete sealing of the microhematocrit tube
	Increased cell shrinkage	Excess EDTA; K_3 EDTA rather than K_2 EDTA or Na_2EDTA
Pseudoincreased MCH	Spuriously high hemoglobin	(See hemoglobin)
	Spuriously low erythrocyte count	(See erythrocyte count)
Pseudodecreased MCH	Spuriously low hemoglobin	(See hemoglobin)
	Spuriously high erythrocyte count	(See erythrocyte count)

★ **TABLE 43-9** *Continued*

Erroneous Parameter	Underlying Problem	Possible Cause
Pseudoincreased MCHC	Spuriously high hemoglobin	(See hemoglobin)
	Spuriously low hematocrit or product of MCV × erythrocyte count	(See hematocrit)
Pseudodecreased MCHC	Spuriously low hemoglobin	(See hemoglobin)
	Spuriously high hematocrit or product of MCV × erythrocyte count	(See hematocrit)
Pseudothrombocytosis, automated	Very small erythrocytes or RBC fragments	Erythrocyte fragments, microcytes (MCV <50 fL), microspherocytes in burn victims
	Cytoplasmic fragments of nucleated cells	Cytoplasmic fragmentation of blasts in acute leukemia or leukemic cells in certain lymphomas
	Microorganisms: bacteria, fungi, or parasites	Bacterial septicemia, infection with *Candida sp.* similar in size to platelets, presence of *Plasmodium falciparum* trophozoites
	Abnormal precipitates	Cryoglobulinemia, cyrofibrinogenemia
Pseudothrombocytopenia, automated	Aggregates counted as single cell or aggregates exceed upper threshold and are not counted	EDTA-induced platelet aggregation
	Platelet satellitism	Platelets surround WBCs in EDTA-induced process, in particular segmented neutrophils
	Very large platelets that exceed upper threshold for platelet count	Abnormally large platelets seen in myeloproliferative neoplasms or myelodysplastic syndromes
	Sample collection issue	Clotted sample
Pseudoincreased reticulocyte count, automated	Inaccurate gating of RBCS	Platelet clumps, very large platelets, WBC fragments
	Other RBC inclusions	Howell-Jolly bodies, Pappenheimer bodies, Heinz bodies, basophilic stippling
	Intraerythrocytic parasites	*Plasmodium spp.*, *Babesia*
	Abnormal precipitates	Cold agglutinins, paraproteinemia
	Autofluorescence of RBCs	Porphyria, certain drugs

EDTA = ethylenediaminetetraacetic acid; HbC = hemoglobin C; CLL = chronic lymphocytic leukemia; HbS = hemoglobin S; RBC = red blood cell; MCV = mean corpuscular volume; K_2 EDTA = dipotassium ethylenediaminetetraacetic acid; K_3 EDTA = tripotassium ethylenediaminetetraacetic acid; Na_2 EDTA = disodium ethylenediaminetetraacetic acid; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; WBCs = white blood cells

[a]Brigden ML, Dalal BI. Spurious and artifactual test results I: Cell counter-related abnormalities. *Lab Med.* 1999;30(5):325–34.

[b]Zandecki M, Genevieve F, Gerard J, Godon A. Spurious counts and spurious results on haematology analysers: A review. Part 1: Platelets. *Int Jnl Lab Hem.* 2007;29:4–20.

[c]Zandecki M, Genevieve F, Gerard J, Godon A. Spurious counts and spurious results on haematology analysers: A review. Part II: White blood cells, red blood cells, haemoglobin, red cell indices and reticulocytes. *Int Jnl Lab Hem.* 2007;29:21–41.

differences have been carefully set, correct results will not be flagged. Therefore, the most likely causes of a delta check is a sample mislabeling or random testing error.

In the hematology laboratory, certain tests, especially the erythrocyte indices, platelet counts, prothrombin time (PT), and other coagulation studies, have very little intraindividual variation. A delta check for one of these parameters should result in an investigation before any results are reported (see Web Table 43-9 for the investigation process).

Correction for Interfering Substances

The presence of lipemia, icterus, or hemolysis in the plasma of an EDTA-anticoagulated blood sample can cause an artificial elevation of the hemoglobin because of increased absorbance of light from the instrument's light source by the diluted sample (Chapter 37). The presence of interfering substances is commonly detected by the application of the Rule of 3, or hemoglobin × 3 = hematocrit ± 3. To correct for the presence of these substances, an aliquot of the well-mixed blood sample is placed in another test tube and centrifuged at 1500 rpm for 5 minutes. A hemoglobin determination is performed on the plasma (supernatant). The corrected hemoglobin is calculated by subtracting the plasma hemoglobin result from the whole blood hemoglobin result ($Hemoglobin_{corrected} = Hemoglobin_{original} - Hemoglobin_{supernatant}$). Alternatively, the plasma can be removed from the centrifuged sample and replaced with an equal volume of saline. The cells are resuspended in the saline, and the corrected hemoglobin is obtained by performing a hemoglobin determination on this sample. The MCH and MCHC should be recalculated using the corrected hemoglobin result and initial red blood cell count and hematocrit regardless of the correction method used for the hemoglobin.

Certain patients develop IgM antibodies or cold agglutinins directed against erythrocyte antigens. As the blood sample cools, these antibodies begin to agglutinate erythrocytes. The automated cell-counting instrument evaluates the agglutinated erythrocytes as one cell, resulting in a decreased erythrocyte count, increased MCV, decreased hematocrit, and MCHC >36 g/dL. Incubation of the blood sample at 37°C for 15 minutes disrupts the antigen–antibody reaction and dissociates the agglutinated erythrocytes. The warmed sample should be mixed thoroughly and analyzed immediately;

its results are reportable unless the platelet count decreases by more than 50 × 10^3/mcL. Warming the sample occasionally causes loss of platelets. If this occurs, the original platelet count should be reported. Some patients exhibit a strong cold agglutinin titer, and the results do not correct after extended warming. In this situation, a manual hematocrit should be performed, and the following additional results should be reported from the original sample: white blood count, hemoglobin, and platelet count. The other CBC parameters cannot be reported.

Hemostasis

As in hematology, patient results for coagulation tests should be reviewed for accuracy. This includes ensuring correct procurement of samples, checking for interfering substances, and using delta checks.

Overanticoagulation/Underanticoagulation

As discussed in Chapter 36, the proper ratio of blood:anticoagulant is 9:1 for the coagulation sample. All sample tubes should be examined visually to ensure that they have been properly filled. Comparison tubes can be prepared by adding water to empty collection tubes up to the expected fill level. Coagulation sample collection tubes containing <90% of the expected volume must be rejected because the sample is overanticoagulated because of a decrease in the 9:1 ratio.

The ratio of blood:anticoagulant is affected when a patient's hematocrit exceeds 55%. The sample has less plasma to be anticoagulated compared to a sample with a normal hematocrit (e.g., 45%). This results in an overanticoagulated sample and falsely prolonged clotting times. The effect on clotting times (i.e., PT and APTT) has been observed with the use of 3.2% sodium citrate as an anticoagulant.[46,47] The correct amount of anticoagulant to use is determined by the formula given in Web Figure 43-5 and is based on the patient's hematocrit.

☑ CHECKPOINT 43-10

The laboratory professional observes that the 3.2% sodium citrate tube for a PT and APTT is only two-thirds full. Explain the effect this will have on the patient's coagulation results.

Interfering Substances

Hemolyzed samples are unacceptable for coagulation testing because thromboplastin-like substances have been released, resulting in shortened clotting times. Coagulation instruments based on photooptical detection could be unable to test samples that contain interfering substances such as lipemia or icteria because these substances affect the endpoint detection (e.g., absorbance). An electromechanical (Chapter 36) or manual clot detection method should be used to obtain accurate results from these samples.

Use of Delta Checks

Each hemostasis laboratory must determine appropriate limits for delta checks. In general, a change in the PT of ±5 seconds or in the APTT of ±15 seconds from a sample tested in the previous 24 hours could indicate a mislabeled sample.

Summary

This chapter reviewed the three components of a laboratory's quality assessment program: pre-examination, examination, and post-examination. The pre-examination component includes all aspects, such as sample collection, handling, and test requisition that could occur before testing and affect the results. The examination component includes all testing aspects including proficiency testing and personnel competency. Proficiency testing monitors the reliability of a laboratory's test results by comparison to those of its peers and provides a good indication of a test method's long-term accuracy. Competency testing ensures that laboratory professionals are proficient in performing, interpreting, and troubleshooting test procedures within their assigned area. The post-examination includes aspects, such as review of patient results and turnaround time, that occur after the testing is performed and could affect the results.

When a new instrument or method is introduced to the laboratory, method evaluation studies must be performed to compare the new method to the current method, assess the new one for random and systematic variation, and validate the reportable range. Precision studies assess random variation, and linear regression analysis assesses systematic variation. A laboratory that chooses to use the manufacturer's reference intervals must validate them to be able to use them for that laboratory and patient population. A laboratory can choose to establish its own reference interval. OSHA and other federal and state agencies mandate safety procedures to which each laboratory must adhere.

The laboratory's quality control program including the use of Westgard rules and moving averages monitors a test method's day-to-day reliability and provides an early indication of potential problems with it. Each laboratory creates its multirule protocol for an instrument by selecting a combination of Westgard rules.

Review of patient results is necessary to ensure that results reflect the patient's condition. This review is a critical component of the quality assessment program. The laboratory professional's ability to recognize and take corrective action when abnormal patient results occur is at the heart of the medical laboratory science profession. Review and recognition of abnormal patient results followed by corrective action represent the final steps before a test result is reported. The physician uses the reported test results to make critical decisions in a patient's diagnosis and treatment or management of disease. Therefore, a good quality assessment program directly affects patient care.

Review Questions

Level I

1. Which source of error represents a pre-examination factor? (Objective 1)
   A. Failure to refrigerate the thromboplastin reagent
   B. Failure to correct the platelet count when using sodium citrate
   C. Failure to invert collection tubes properly
   D. Failure to perform daily maintenance on cell-counting instrument
2. Under CLIA '88, how frequently should proficiency testing be performed? (Objective 4)
   A. once a year
   B. twice a year
   C. three times a year
   D. four times a year
3. When validating a manufacturer's reference interval, what is the minimum number of subject samples that should be used? (Objective 5)
   A. 10
   B. 20
   C. 60
   D. 120
4. Which governmental organization implemented universal precautions? (Objective 6)
   A. OSHA
   B. CDC
   C. FDA
   D. CMS
5. Which of the following is an OSHA recommendation to be followed in the clinical laboratory? (Objective 7) (Refer to Web Table 43-7)
   A. Gloves should be worn when working with blood but are not required when performing a venipuncture.
   B. Safe needle devices should be used when performing a venipuncture or capillary puncture.
   C. Mouth pipetting is acceptable for transferring liquid reagents (e.g., saline or deionized water).
   D. Hand washing is necessary only if hands become contaminated with blood or other body fluids.
6. Which critical information regarding a chemical is found on its MSDS? (Objective 8)
   A. expiration date
   B. lot number
   C. storage requirements
   D. intended use
7. You are responsible for determining the control limits for the low fibrinogen control. The data points were collected, and the mean ($x = 64$ mg/dL) and SD ($s = 3.0$ mg/dL) were determined. What are the 3SD limits? (Objective 10)
   A. 61.0–67.0 mg/dL
   B. 58.0–70.0 mg/dL
   C. 55.0–73.0 mg/dL
   D. 52.0–76.0 mg/dL
8. How should the laboratory professional interpret the PT control results for the current control run if the ± 2 SD control limits are 11.80–14.20 seconds for level I and 25.00–28.00 seconds for level II? (Objective 11)

Level I	11.6 seconds
Level II	24.6 seconds

   A. as acceptable
   B. as unacceptable
9. Which of the following parameters will the presence of lipemia affect? (Objective 13)
   A. RBC, Hb, Hct, MCV, MCH, MCHC
   B. RBC, Hct, MCV, MCHC
   C. Hb, MCH, MCHC
   D. Hct, MCV, MCHC
10. In interpreting a patient's CBC results, the laboratory professional notes that the platelet count is 25×10^3/mcL. Which of the following could be associated with this finding? (Objective 12)
    A. observation of erythrocyte fragments on the blood smear
    B. presence of cryoglobulins
    C. observation of platelet clumps at the feather edge of the blood smear
    D. presence of intracellular parasites such as malaria

Level II

1. Which is the first step in selecting a new instrument? (Objective 3)
   A. conducting a needs assessment survey
   B. determining the cost per test
   C. performing an in-house evaluation of the instrument
   D. evaluating the quality control program
2. Which performance study in the method evaluation process assesses random variation? (Objective 3)
   A. precision study
   B. method comparison study
   C. linearity study
   D. calibration study
3. Given the following linear regression results, which is the appropriate interpretation? (Objective 2)

y-Intercept	0.869
Slope	1.027
Standard error of the estimate	0.014

   A. No significant difference exists between the new method and current method results.
   B. A proportional systematic error exists between the new method and current method results.
   C. A random error exists between the new method and current method results.
   D. A constant systematic error exists between the new method and current method results.
4. To verify the hemoglobin reportable range on an automated hematology instrument, the laboratory's first step is to: (Objective 4)
   A. calibrate the instrument and perform quality control
   B. select the statistical method for data analysis
   C. determine the laboratory's goal for nonlinear error
   D. choose linearity check materials that span the instrument's reportable range
5. After performing daily quality control on the automated hematology instrument, the laboratory professional observes a 2_{2s} violation for the hemoglobin, MCH, and MCHC parameters. All other parameters were in control. What type of error is indicated? (Objective 5)
   A. random
   B. systematic
6. Regarding question 5, which is the most likely cause of the error? (Objective 5)
   A. failure to adequately mix the control materials
   B. improper storage of the control materials
   C. depleted supply of lysing reagent
   D. expired diluting fluid
7. For the automated hematology instruments, moving averages of erythrocyte indices can be used to monitor the instrument's performance in determining which parameter: (Objective 6)
   A. leukocyte
   B. erythrocyte
   C. platelet
   D. reticulocyte
8. Initial interpretation of a patient's CBC results showed an MCHC of 38 g/dL. The laboratory professional warmed the sample to 37°C and retested it. No change was observed. The appropriate course of action is to: (Objective 8)
   A. observe the blood smear for the presence of spherocytes
   B. replace the patient's plasma with an equal volume of saline and then reanalyze
   C. recollect patient sample using sodium citrate and analyze it
   D. examine the blood smear for the presence of nucleated erythrocytes
9. The following coagulation test results were obtained: PT = 7.5 seconds and APTT = 20 seconds. How should the laboratory professional approach these findings? (Objective 8)
   A. report the results to the patient's chart
   B. observe the sample for the presence of hemolysis
   C. check the sample for small clots
   D. examine the collection tube to determine whether it was underfilled
10. Which source of error would a delta check detect? (Objective 10)
    A. depleted reagent supply
    B. improper calibration of the instrument
    C. deteriorating light source
    D. failure to correctly label the patient's sample

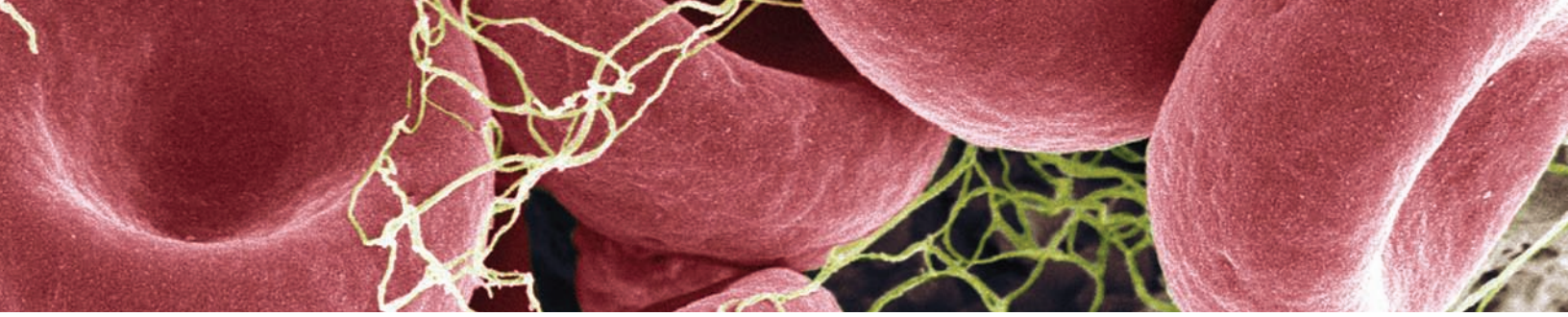
Companion Resources

http://www.pearsonhighered.com/healthprofessionsresources/

The reader is encouraged to access and use the companion resources created for this chapter. Find additional information to help organize information and figures to help understand concepts.

References

1. Centers for Disease Control and Prevention (CDC), Centers for Medicare & Medicaid Services (CMS), HHS. Medicare, Medicaid, and CLIA programs: laboratory requirements relating to quality systems and certain personnel qualifications. Final rule. *Fed Regist*. 2003;68(16):3639–714.
2. Barth JH. Clinical quality indicators in laboratory medicine. *Ann Clin Biochem*. 2012;49:1–16.
3. Carver CJ. Establishing a thorough quality assurance program. *Adv Med Lab Professionals*. 2000;12(9):6.
4. Lippi G. Governance of preanalytical variability: traveling the right path to the bright side of the moon? *Clin Chim Acta*. 2009;404:32–36.
5. Stankovic AK, Smith S. Elevated serum potassium values: the role of preanalytical variables. *Am J Clin Pathol*. 2004;121(suppl 1):S105–12.
6. King D. A variety of variables. *Adv Med Lab Professionals*. 2000;12(24):14–17.
7. Clinical and Laboratory Standards Institute. *Laboratory Documents: Development and Control; Approved Guideline*, 5th ed. Wayne, PA: CLSI; 2006. GP2-A5.
8. Medicare, Medicaid, and CLIA programs: laboratory requirements relating to quality systems and certain personnel qualifications. *Fed Regist*. 2003;68(16):3706. Final Rule, 42 CFR Part 493, Subpart K (493.1251).
9. Miller WG, Jones G, Horowitz GL et al. Proficiency testing/external quality assessment: current challenges and future directions. *Clin Chem*. 2011;57:1670–680.
10. Carver C. A closer look at post-analytical policies and procedures. *Adv Med Lab Professionals*. 2000;12(7):6.
11. Piva E, Sciacovelli L, Zaninotto M et al. Evaluation of effectiveness of a computerized notification system for reporting critical values. *Am J Clin Pathol*. 2009;131:432–41.
12. Centers for Disease Control and Prevention. HIPAA privacy rule and public health. Guidance from CDC and the U.S. Department of Health and Services. *MMWR Morb Mortal Wkly Rep*. 2003;52(suppl):1–17, 19–20.
13. Medicare, Medicaid, and CLIA programs: laboratory requirements relating to quality systems and certain personnel qualifications. *Federal Register*. 2003;68(16):3702–3703. Final Rule, 42 CFR Part 493, Subpart H (493.800) and Subpart I (493.900).
14. Ehrmeyer SS, Laessig RH. Has compliance with CLIA requirements really improved quality in US clinical laboratories? *Clin Chim Acta*. 2004;346(1):37–43.
15. Medicare, Medicaid, and CLIA programs: laboratory requirements relating to quality systems and certain personnel qualifications. Final Rule, 42 CFR Part 493, Subpart K (493.1235). *Fed Regist*. 2003;68(16):3705.
16. Clinical Laboratory Improvement Amendments of 1988. Final Rule, 42 CFR Part 493, Subpart M (493.1413). *Fed Regist*. 1992;57(40):7174.
17. Leach AP, Haun DE. Assessing competence in finding and reporting abnormal morphologic features while scanning peripheral blood smears. *Clin Lab Sci*. 2000;13(3):160–65.
18. Paxton A. CAP program takes competency assessment paperless. *CAP Today*. 2009;23(2):1–4.
19. Mina A, Favaloro EJ, Koutts J. A practical approach to instrument selection, evaluation, basic financial management and implementation in pathology and research. *Clin Chem Lab Med*. 2008;46:1223–229.
20. Barglowski M. The instrument selection process: beyond the sales pitch. *MLO Med Lab Obs*. 2001;33(2):45–51.
21. Polancic J, Roncancio G. *Method Verification and Instrument Selection Procedures*. Springfield: The Laboratory Consultants, Inc. of Illinois; October 1994.
22. Clinical and Laboratory Standards Institute. *Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline*, 2nd ed. Wayne, PA: CLSI, 2010. EP09-A2-IR. Interim revision.
23. McCudden CR, Rogers M, Erickson J et al. Method evaluation and quality management. In: Bishop ML, Fody EP, Schoeff L, eds. *Clinical Chemistry: Techniques, Principles, Correlations*, 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2010.
24. Medicare, Medicaid, and CLIA programs: laboratory requirements relating to quality systems and certain personnel qualifications. *Fed Regist*. 2003;68(16):3707. Final Rule, 42 CFR Part 493, Subpart K (493.1253).
25. Clinical and Laboratory Standards Institute. *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline*. Wayne, PA: CLSI; 2003. EP6-A.
26. Clinical and Laboratory Standards Institute. *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline*, 3rd ed. Wayne, PA: CLSI; 2008. C28-A3c.
27. Winsten S. The ecology of normal values in clinical chemistry. *Crit Rev Clin Lab Sci*. 1976;6:319–30.
28. Centers for Disease Control. Acquired immunodeficiency syndrome (AIDS): precautions for clinical and laboratory staffs. *MMWR Morb Mortal Wkly Rep*. 1982;31:577–80.
29. Panlilio AL, Cardo DM, Grohskopf LA et al. US Public Health Service. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HIV and recommendations for postexposure prophylaxis. *MMWR Recomm Rep*. 2005;54(RR-9):1–17.
30. Clinical and Laboratory Standards Institute. *Clinical Laboratory Safety; Approved Guideline*, 3rd ed. Wayne, PA: CLSI; 2012. GP17-A3.
31. Occupational Safety and Health Administration hazard communication. Final Rule, 29 CFR. *Fed Regist*. 1994;59:6126–84.
32. Westgard JO. QC: The calculations. In: Westgard JO, ed. *Basic QC Practices: Training in Statistical Quality Control for Healthcare Laboratories*, 3rd ed. Madison, WI: Westgard Quality Corporation; 2010.
33. Westgard JO, Barry PL, Hunt MR et al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem*.1981;27:493–501.
34. Westgard JO. QC: The multirule procedure and multilevel interpretation. In: Westgard JO, ed. *Basic QC Practices: Training in Statistical Quality Control for Healthcare Laboratories*, 3rd ed. Madison, WI: Westgard Quality Corporation; 2010.
35. Bull BS, Elashoff RM, Heilbron DC et al. A study of various estimators for the derivation of quality control procedures from patient erythrocyte indices. *Am J Clin Pathol*. 1974;61:473–81.
36. *CELL-DYN Sapphire™ Operator's Manual*. Abbott Park, IL: Abbott; November 2007:9140415C.
37. Clinical and Laboratory Standards Institute. *Autoverification of Clinical Laboratory Test Results; Approved Guideline*. Wayne, PA: CLSI; 2006. AUTO10-A.
38. Cornbleet J. Spurious results from automated hematology cell counters. *Lab Med*. 1983;14(8):509–14.
39. Brigden ML, Dalal BI. Spurious and artifactual test results I: cell counter-related abnormalities. *Lab Med*. 1999;30(5):325–34.
40. Zandecki M, Genevieve F, Gerard J et al. Spurious counts and spurious results on haematology analysers: a review. Part 1: platelets. *Int J Lab Hematol*. 2007;29:4–20.
41. Zandecki M, Genevieve F, Gerard J et al. Spurious counts and spurious results on haematology analysers: a review. Part II: white blood cells, red blood cells, haemoglobin, red cell indices and reticulocytes. *Int J Lab Hematol*. 2007;29:21–41.
42. Tessier-Marteau A, Genevieve F, Godon A et al. Automated hematology analysers and spurious counts. Part 1: platelets. *Ann Biol Clin*. 2010; 68(4):393–407.
43. Genevieve F, Godon, A, Tessier-Marteau A et al. Automated hematology analysers and spurious counts. Part 2: leukocyte count and differential. *Ann Biol Clin*. 2012; 70(2):141–54.
44. Godon A, Genevieve F, Tessier-Marteau A et al. Automated hematology analysers and spurious counts. Part 3: haemoglobin, red blood cells, cell count and indices, reticulocytes. *Ann Biol Clin*. 2012;70(2):155–68.
45. Houwen B, Duffin D. Delta checks for random error detection in hematology tests. *Lab Med*. 1989:20(6):378–81.
46. Marlar RA, Potts RM, Marlar AA. Effect on routine and special coagulation testing values of citrate anticoagulant adjustment in patients with high hematocrit values. *Am J Clin Pathol*. 2006:126:400–05.
47. Favaloro EJ, Funk DM, Lippi G. Pre-analytical variables in coagulation testing associated with diagnostic errors in hemostasis. *Lab Med*. 2012;43(2):1–10.



Appendix A: Answers to Review Questions

Chapter 1

1. a
2. d
3. a
4. c
5. b
6. a
7. a
8. a

Chapter 2

Level I

1. b
2. c
3. d
4. c
5. a
6. a
7. b
8. c
9. a
10. d

Level II

1. d
2. b
3. c
4. b
5. a
6. d
7. a
8. d
9. b
10. b

Chapter 3

Level I

1. a
2. b
3. a
4. d
5. a

Level II

1. a
2. a
3. d
4. a
5. b
6. d
7. c

Chapter 4

Level I

1. b
2. c
3. d
4. a
5. d
6. a
7. b
8. c
9. b
10. c

Level II

1. c
2. a
3. c
4. b
5. a
6. d
7. d
8. a
9. b
10. c

Chapter 5

Level I

1. a
2. b
3. b
4. c
5. a

6. c
7. d
8. d
9. c
10. b

Level II

1. d
2. d
3. c
4. d
5. d
6. b
7. c
8. a
9. a
10. c

Chapter 6

Level I

1. a
2. b
3. b
4. c
5. d
6. c
7. b
8. c
9. b
10. d

Level II

1. d
2. b
3. a
4. c
5. b
6. d
7. b
8. b
9. a
10. a

Chapter 7

Level I

1. c
2. b
3. b
4. a
5. a
6. d
7. d
8. a
9. c
10. a
11. c
12. a
13. b
14. b
15. d

Level II

1. a
2. d
3. a
4. c
5. b
6. a
7. d
8. d
9. d
10. a
11. c
12. a
13. d
14. d
15. a

Chapter 8

Level I

1. b
2. b
3. d
4. c
5. c
6. b
7. c
8. a
9. b
10. b

Level II

1. c
2. b
3. d
4. a
5. b
6. c
7. c
8. c
9. d
10. b

Chapter 9

Level I

1. d
2. c
3. b
4. c
5. a
6. d
7. c

Level II

1. c
2. a

3. c
4. d
5. b
6. c

Chapter 10

Level I

1. b
2. c
3. b
4. b
5. b
6. c
7. d
8. c
9. b
10. c

Level II

1. b
2. a
3. a
4. c
5. c
6. c
7. c
8. d
9. b
10. b

Chapter 11

Level I

1. d
2. a
3. a
4. a
5. d
6. a
7. d
8. c
9. b
10. c

Level II

1. c
2. d
3. c
4. d
5. a
6. c
7. d
8. b
9. a
10. d
11. d
12. b

Chapter 12

Level I

1. b
2. a
3. c
4. c
5. d
6. d
7. d
8. b
9. d
10. c

Level II

1. a
2. d
3. b
4. c
5. b
6. c
7. b
8. a
9. b
10. a
11. c
12. c

Chapter 13

Level I

1. a
2. d
3. c
4. c
5. a
6. c
7. c
8. d
9. d
10. c

Level II

1. b
2. d
3. a
4. c
5. b
6. a
7. b
8. d
9. b
10. a

Chapter 14

Level I

1. c
2. a
3. d

4. a
5. d
6. c
7. b
8. a
9. c
10. b

Level II

1. a
2. c
3. a
4. a
5. d
6. b
7. b
8. d
9. a
10. a

Chapter 15

Level I

1. b
2. a
3. c
4. c
5. d
6. a
7. b
8. c
9. a
10. c

Level II

1. b
2. d
3. c
4. b
5. a
6. a
7. b
8. d
9. c
10. b

Chapter 16

Level I

1. a
2. d
3. c
4. a
5. b
6. b
7. a
8. d
9. a
10. c

Level II

1. d
2. d
3. a
4. b
5. c
6. c
7. b
8. c
9. d
10. a

Chapter 17

Level I

1. a
2. c
3. d
4. b
5. b
6. d
7. c
8. c
9. d
10. b

Level II

1. c
2. b
3. c
4. a
5. a
6. c
7. a
8. d
9. c
10. b

Chapter 18

Level I

1. d
2. b
3. c
4. d
5. c
6. a
7. a
8. c
9. a
10. d

Level II

1. c
2. b
3. a
4. b
5. a
6. c
7. a
8. d

9. d
10. a

Chapter 19

Level I

1. b
2. c
3. a
4. a
5. d
6. c
7. c
8. b
9. c
10. b

Level II

1. b
2. b
3. a
4. a
5. d
6. d
7. c
8. a
9. c
10. d

Chapter 20

Level I

1. b
2. d
3. a
4. d
5. c
6. d
7. a
8. a
9. c
10. a

Level II

1. a
2. c
3. d
4. b
5. a
6. c
7. d
8. b
9. c
10. d

Chapter 21

Level I

1. b
2. d
3. a
4. b
5. a
6. a
7. c
8. a
9. c
10. c

Level II

1. d
2. c
3. d
4. b
5. c
6. a
7. b
8. d
9. b
10. a
11. c
12. a

Chapter 22

Level I

1. c
2. b
3. c
4. d
5. b
6. c
7. c
8. d
9. d
10. c

Level II

1. a
2. a
3. c
4. c
5. b
6. a
7. d
8. b
9. a
10. d

Chapter 23

Level I

1. a
2. a
3. b
4. b
5. b
6. a
7. b
8. a

9. d
10. b
11. a
12. d
13. a
14. d

Level II

1. a
2. c
3. d
4. c
5. a
6. b
7. c
8. b
9. d
10. b
11. a
12. c
13. b
14. c
15. d

Chapter 24

Level I

1. b
2. a
3. b
4. c
5. a
6. b
7. d
8. a
9. c
10. b

Level II

1. b
2. a
3. c
4. b
5. c
6. d
7. a
8. a
9. d
10. b

Chapter 25

Level I

1. a
2. c
3. d
4. a
5. c
6. c
7. d
8. a
9. b
10. c

Level II

1. d
2. b
3. a
4. b
5. a
6. d
7. b
8. a
9. b
10. c

Chapter 26

Level I

1. b
2. a
3. b
4. b
5. a
6. c
7. a
8. c
9. d
10. d

Level II

1. a
2. d
3. a
4. c
5. a
6. a
7. a
8. a
9. d
10. d

Chapter 27

Level I

1. b
2. d
3. c
4. b
5. b
6. d
7. a
8. c
9. c
10. b

Level II

1. d
2. d
3. a
4. a
5. b

6. c
7. b
8. a
9. b
10. a

Chapter 28

Level I

1. d
2. b
3. d
4. d
5. c
6. a
7. d
8. c
9. d
10. a

Level II

1. a
2. c
3. a
4. c
5. a
6. b
7. a
8. a
9. a
10. a

Chapter 29

Level I

1. d
2. d
3. b
4. c
5. b
6. a
7. a
8. d
9. c
10. b

Level II

1. c
2. c
3. a
4. d
5. d
6. d
7. b
8. b
9. c
10. a

Chapter 30

Level I

1. d
2. c
3. d
4. d
5. a
6. d
7. d
8. a
9. d
10. a

Level II

1. d
2. a
3. b
4. a
5. b
6. a
7. c
8. a
9. c
10. c
11. d

Chapter 31

Level I

1. b
2. c
3. c
4. c
5. d
6. d
7. d
8. b
9. a
10. a

Level II

1. b
2. a
3. d
4. a
5. c
6. d
7. a
8. b
9. c
10. b

Chapter 32

Level I

1. b
2. a
3. c
4. c
5. b
6. b
7. b

8. c
9. c
10. a

Level II

1. a
2. d
3. d
4. c
5. b
6. b
7. d
8. a
9. b
10. a

Chapter 33

Level I

1. d
2. d
3. d
4. b
5. a
6. c
7. d
8. d
9. d
10. b

Level II

1. c
2. a
3. c
4. a
5. b
6. d
7. c
8. d
9. c
10. a

Chapter 34

Level I

1. d
2. a
3. b
4. b
5. c
6. d
7. a
8. d
9. b
10. b

Level II

1. b
2. a
3. c
4. c
5. b
6. a
7. c
8. b
9. d
10. b

Chapter 35

Level I

1. a
2. a
3. c
4. d
5. b
6. d
7. d
8. c
9. a
10. a

Levelv II

1. a
2. c
3. b
4. d
5. a
6. a
7. c
8. d
9. a
10. b

Chapter 36

Level I

1. b
2. c
3. b
4. a
5. a
6. b
7. b
8. a
9. c
10. a
11. c
12. b
13. d
14. a

Level II

1. c
2. a
3. d
4. c
5. a

6. d
7. c
8. b
9. b
10. a
11. a

Chapter 37

Level I

1. c
2. c
3. d
4. a
5. b
6. b
7. d
8. a
9. c
10. a

Level II

1. b
2. d
3. a
4. b
5. a
6. b
7. a
8. c
9. a
10. b

Chapter 38

Level I

1. c
2. a
3. b
4. d
5. a
6. a
7. c
8. b
9. a
10. a

Level II

1. b
2. c
3. c
4. a
5. a
6. c
7. b
8. a
9. c
10. b

Chapter 39

Level I

1. d
2. a
3. d
4. b
5. b
6. d
7. c
8. b
9. d
10. c

Level II

1. a
2. b
3. c
4. d
5. a
6. d
7. c
8. c
9. b
10. a

Chapter 40

Level I

1. d
2. c
3. d
4. d
5. d
6. c
7. d
8. b
9. a
10. b

Level II

1. b
2. a
3. b
4. d
5. c
6. b
7. d
8. a
9. d
10. b

Chapter 41

Level I

1. a
2. c

3. c
4. a
5. a
6. a
7. a
8. b
9. c
10. d

Level II

1. b
2. d
3. c
4. c
5. c
6. a
7. b
8. a
9. d
10. d

Chapter 42

Level II

1. c
2. b
3. a
4. b
5. a
6. c
7. b
8. d
9. d
10. d

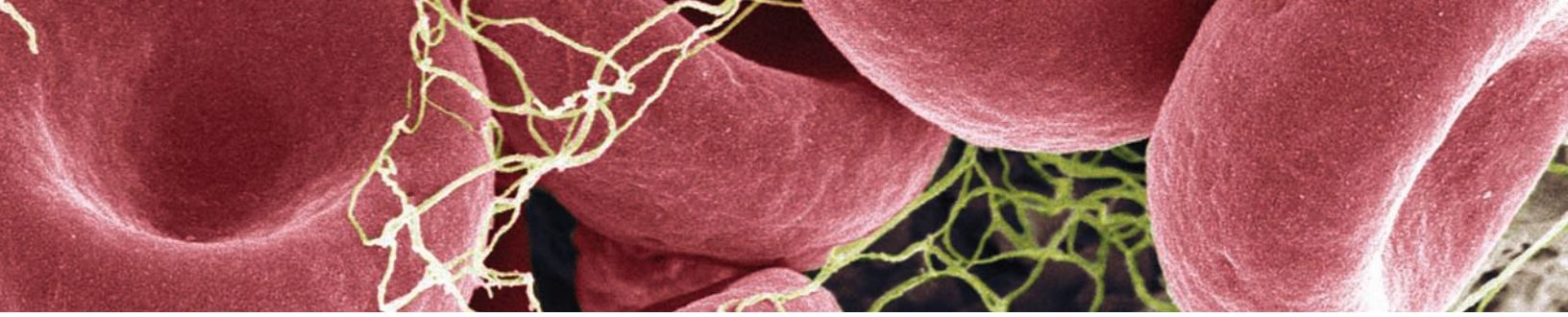
Chapter 43

Level I

1. c
2. c
3. b
4. b
5. b
6. c
7. c
8. b
9. c
10. c

Level II

1. a
2. a
3. d
4. c
5. b
6. c
7. b
8. a
9. b
10. d



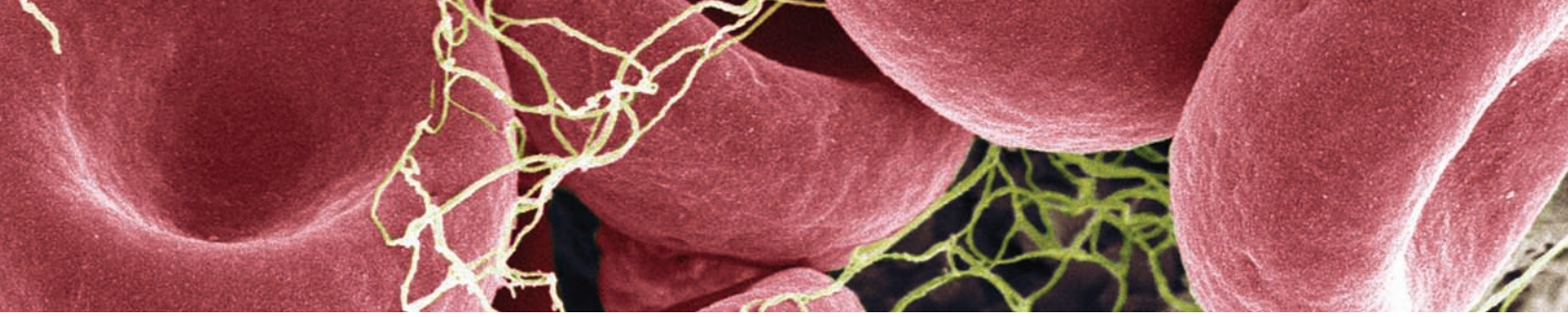
Appendix B: Hematopoietic and Lymphoid Neoplasms: Immunophenotypic and Genetic Features

Disease Category	Diagnosis	Immunophenotype	Chromosome Translocation	Genotypic Finding
Mature B-cell neoplasms	Chronic lymphocytic leukemia/small lymphocytic lymphoma	CD19+, CD20+dim, CD5+, CD10−, CD23+, FMC-7−, sIg+dim	No specific abnormality; could have del(13q14.2), trisomy 12, del(11q21), del(17p13.1), del(6q21) t(14;19)(q32.3;19q13)	No specific abnormality; subtypes with and without somatic hypermutation *IGHV*
	B-cell prolymphocytic leukemia	CD19+, CD20+, CD5+/−, CD23+/−, FMC-7+	No specific abnormality; complex karyotype and del(17p.1) frequent	No specific abnormality; *TP53* mutations frequent
	Hairy cell leukemia	CD19+, CD10−, CD5−, CD11c+, CD25+, CD103+, annexin-A1+, sIg+	No specific abnormality	*BRAF* mutation in most
	Follicular lymphoma	CD19+, CD10+, CD5−, bcl-2+, sIg+	t(14;18)(q32.3;q21.3)	*IGH@/BCL2* rearrangement
	Mantle cell lymphoma	CD19+, CD10−, CD5+, CD23+/−, FMC-7+, cyclin-D1+, sIg+	t(11;14)(q13;q32.3)	*CCND1/IGH@* rearrangement
	Extranodal marginal zone lymphoma of mucosa associated lymphoid tissue type (MALT lymphoma)	No specific phenotype CD19+, CD20+, CD5−, CD10−, sIg+; could have plasmacytic differentiation	Variety of associated translocations that vary in frequency among sites t(11;18)(q22;q21), t(1;14)(p22;q32.3), t(14;18)(q32.3;q21), t(3;14)(p14.1;q32.3), +3, +18	No specific abnormality; *BIRC3/MALT1* fusion, *or BCL10, MALT1, FOXP1;* transcriptional deregulation
	Lymphoplasmacytic lymphoma	No specific phenotype CD19+, CD20+, CD5−, CD10−, sIg+ lymphoid cells and plasmacytic differentiation	No specific abnormality; often del(6q)	No specific abnormality; *MYD88 L265*
	Diffuse large B-cell lymphoma	No specific phenotype CD19+, CD20+, CD5+/−, CD10+/−	No specific abnormality; could have abnormalities of 3q27, t(14;18)(q32.3;q21)	No specific abnormality; could have *BCL6, BCL2, or MYC* rearrangement, often with *IGH.*
	Burkitt lymphoma	CD19+, CD10+, CD5−, sIg+	t(8;14)(q24.1;q32.3) or t(8;22)(q24.1;q11.2), t(2;8)(p11.2;q24.1)	*MYC/IGH@, IGK@,* or *IGL@*

Disease Category	Diagnosis	Immunophenotype	Chromosome Translocation	Genotypic Finding
	Plasma cell neoplasms	CD138+, CD38+bright, cIg+, often with CD56+, CD19−	No specific abnormality; often translocations involving 14q32.3 and cyclin-D1 (11q13), *MAF* (16q22-q23), *FGFR3/WHSC1* (4p16.3), cyclin-D3 (6p21), and *MAFB* (20q11.1-q13.1)	No specific abnormality *IGH@ and CCND1*, *MAF*, *FGFR3/WHSC1*, *MAFB* often rearranged
Mature T-cell neoplasms	T-cell prolymphocytic leukemia	No specific phenotype. CD2+, CD3+, CD7+, CD4+/−, CD8+/−, TCL1+.	inv(14)(q11.2q32.3), t(14;14)(q11.2;q32.3), t(X;14)(q28;q11.2); also, idic(8p11), t(8;8)(p11-12;q12), +(8q), and del(12p13)	No specific abnormality; often *TRA@* juxtaposed with *TCL1A* and *TCL1B* or *MTCP1*; *ATM* mutation; often *TP53* mutation
	T-cell large granular lymphocytic leukemia	CD3+, usually CD8+, often with decreased expression of CD5 or CD7, CD57+, CD16+	No specific abnormality	No specific abnormality
	Sézary syndrome	CD3+, CD4+, CD5+, CD7−	No specific abnormality; complex karyotypes common	No specific abnormality; often *TP53* mutation
	Anaplastic large cell lymphoma	Could express T-cell antigens (e.g., CD2, CD3, CD5, CD7), but often "null" phenotype lacking antigens	t(2;5)(p23;q35.1), or t(1;2)(q21.2;p23), or inv(2)(p23q35), or others	*ALK* rearranged with *NPM1*, *TPM3*, *ATIC*, or others
	Peripheral T-cell lymphoma, NOS	No specific phenotype CD3+, CD4+/−, CD8−/+, CD5+/−, CD7+/−	No specific abnormality; complex karyotypes common	No specific abnormality
Precursor lymphoid neoplasms	B lymphoblastic leukemia/lymphoma (B LL) with t(9;22)(q34.1;q11.2)	CD19+, CD10+, CD34+, TdT+, often with aberrant expression of CD13 and/or CD33	t(9;22)(q34.1;q11.2)	*BCR-ABL1* usually in minor breakpoint cluster region leading to p190 fusion protein
	B LL with t(v;11q23)	CD19+, CD10−, CD15+, TdT+	11q23 breakpoint	*MLL* rearranged
	B LL with t(12;21)(p13;q22.3)	CD19+, CD10+, CD34+, TdT+, often with aberrant expression of CD13	t(12;21)(p13;q22.3)	*ETV6-RUNX1*
	Hyperdiploid B LL (54-65 chr.)	CD19+, CD10+, CD34+, CD45−	Gain of chromosomes 4, 10, 17, 21, and X most common	Genetic gain related to chromosomes involved
	Hypodiploid B LL (<44 chr.)	CD19+, CD10+	Modal chromosome number from 45 to near-haploid	Genetic loss related to chromosomes involved
	B LL with t(5;14)(q31;q32.3)	CD19+, CD10+	t(5;14)(q35.1;q32.3)	*IL3/IGH@*
	B LL with t(1;19)(q23.3;p13.3)	CD19+, CD10+, Cμ+	t(1;19)(q23.3;p13.3)	*PBX1/TCF3*
Acute myeloid leukemia (AML)	AML with t(8;21)(q22;q22.3)	CD13+, CD33+/−, MPO+, CD34+, HLA-Dr+, aberrant CD19+ blasts in addition to maturing granulocytes	t(8;21)(q22;q22.3)	*RUNX1T1/RUNX1*
	AML with inv(16)(p13.11q22.1) or t(16;16)(p13.11;q22.1)	CD13+, CD33+, MPO+, CD34+, HLA-Dr+ blasts, often in addition to maturing granulocytes or monocytes	inv(16)(p13.11;q22.1) or t(16;16)(p13.11;q22.1)	*CBFB/MYH11*
	Acute promyelocytic leukemia	CD13+, CD33, MPO+, CD34−, HLA-Dr− immature cells	t(15;17)(q24.1;q21.1); Also the following variants: t(11;17)(q23;q21.1) t(5;17)(q35.1;q21.1) t(11;17)(q13;q21.1) dup(17q)	*PML/RARA;* Also the following variants: *ZBTB16/RARA* *NPM1/RARA* *NUMA1/RARA* *STAT5B/RARA*

(*continued*)

Disease Category	Diagnosis	Immunophenotype	Chromosome Translocation	Genotypic Finding
	AML with t(9;11)(p22;q23)	CD13+, CD33+, MPO+, CD34−, HLA-Dr+ blasts, in addition to monocytic cells	t(9;11)(p22;q23)	*MLLT3/MLL*
	AML with t(6;9)(p23;q34)	No specific phenotype	t(6;9)(p23;q34)	*DEK/NUP214*
	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26,2)	No specific phenotype Expression of CD41 has been reported	inv(3)(q21q26.2) t(3;3)(q21;q26.2)	Abnormalities *EVI* *RPN1/EVI1*
	AML with t(1;22)(p13;q13)	CD41 and/or CD61	t(1;22)(p13;q13)	*RBM15/MKL1*
	AML with mutated *NPM1*	Usually lack CD34. Frequently express monocytic antigens CD14 or CD11b	Most have normal karyotype Occasionally +8 and del(9q)	*NPM1* mutation FLT3-ITD ~40%
	AML with mutated *CEBPA*	No specific phenotype	Most have normal karyotype	*CEBPA* mutation FLT3-ITD ~20–30%
	AML with myelodysplasia-related changes	No specific phenotype	−5/del(5q), −7/del(7q), complex karyotype, and others	Multiple genes
	Therapy-related AML/MDS	No specific phenotype	del(5), del(7), complex karyotype, and others 11q23 and 21q22.3 aberrations	Alkylating agent/radiation related *MLL* and *RUNX1* rearranged (topoisomerase-II inhibitor related)
Myelodysplastic syndromes	RCUD RARS RCMD RAEBI, RAEBII MDS, unclassifiable del(5q)	Heterogeneous: lymphoid antigens on myeloid cells, over- or underexpression of antigens, immature/mature antigens on mature/immature cells	Heterogeneous: −5/5q−, −7/7q−, and complex karyotype most frequent cytogenetic aberrations Normal karyotype seen in 50% of cases 5q− only	Heterogeneous: *AXL1, EGR1, EVI1, MDS/EVI1, FLT3, JAK2, NPM1, NRAS/KRAS, RPS14, TP53*
Myeloproliferative neoplasms	Chronic myelogenous leukemia, *BCR-ABL1* positive	Maturing neutrophilic cells and increased basophils Could see nonspecific aberrant antigen expression	t(9;22)(q34.1;q11.2), Philadelphia chromosome (Ph) Disease progression associated with an extra Ph, +8, +19, or i(17q)	*BCR-ABL1* usually in major breakpoint cluster region leading to p210 fusion protein
	Polycythemia vera	Could see nonspecific aberrant antigen expression	No specific cytogenetic abnormality; could see +8, +9, del(20q), del(13q), and del(9p)	*JAK2 V617F* mutation (also seen in other myeloproliferative neoplasms)
	Primary myelofibrosis	No specific abnormality identified	No specific cytogenetic abnormality del(13)(q12-22) or der(6)t(1;6)(q21-q23;p21.3) suggestive of PMF; could see del(20q) , partial trisomy 1q, +9, +8	*JAK2 V617F* mutation in ~50%; *MPL W515K/L* mutation in ~5%
	Essential thrombocythemia	Could see nonspecific aberrant antigen expression	Could see del(20q), +8, abnormalities of 9q	*JAK2 V617F* mutation in ~40–50%; *MPL W515K/L* mutation in ~1%.
Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA, PDGFRB* or *FGFR1*	Myeloid or lymphoid neoplasms with *PDGFRA* rearrangement; myeloid neoplasms with *PDGFRB* rearrangement; myeloid or lymphoid neoplasms with *FGFR1* abnormalities	No specific abnormality identified. Could see aberrant mast cell expression of CD2 and CD25	Cryptic del(4)(q11-q12), t(1;4)(q44;q12), t(4;10(q12;p11); t(5;12)(q33.1;p12) translocations with 8p12	*FIP1L1-PDGFRA* fusion *PDGFRB* rearrangement; variety of *FGFR1* fusion genes



Appendix C: 2008 WHO Classification of Hematologic, Lymphopoietic, Histiocytic/ Dendritic Neoplasms

I. Mature Myeloid Neoplasms

1. Myeloproliferative Neoplasms (MPN)

Chronic myelogenous leukemia (CML), Philadelphia chromosome, (9;22)(q34;q11), *BCR/ABL1* positive

Chronic neutrophilic leukemia (CNL)

Essential thrombocythemia (ET)

Polycythemia vera (PV)

Primary myelofibrosis (PMF)

Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS)

Mastocytosis

- Cutaneous mastocytosis
- Systemic mastocytosis
- Mast cell leukemia
- Mast cell sarcoma
- Extracutaneous mastocytoma

Myeloproliferative neoplasm, unclassifiable (MPN, U)

2. Myeloid and Lymphoid Neoplasms with Eosinophilia and Abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*

Myeloid and lymphoid neoplasms with *PDGFRA* rearrangement

Myeloid neoplasms with *PDGFRB* rearrangement

Myeloid and lymphoid neoplasms with *FGFR1* abnormalities

3. Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPN)

Chronic myelomonocytic leukemia (CMML)

Atypical chronic myeloid leukemia, *BCR/ABL1* negative (aCML)

Juvenile myelomonocytic leukemia (JMML)

Myelodysplasatic/myeloproliferative neoplasm, unclassifiable (MDS/MPN,U)

Provisional entity

- *Refractory anemia with ring sideroblasts and marked thrombocytosis*

4. Myelodysplastic Syndromes (MDS)

Refractory cytopenia with unilineage dysplasia

- Refractory anemia (RA)
- Refractory neutropenia (RN)
- Refractory thrombocytopenia (RT)

Refractory anemia with ring sideroblasts (RARS)

Refractory cytopenia with multilineage dysplasia (RCMD)

Refractory anemia with excess blasts (RAEB)

MDS associated with isolated del(5q)

MDS, unclassifiable (MDS, U)

Childhood MDS

Provisional

Refractory cytopenia of childhood

II. Acute Myeloid Leukemia (AML) and Related Precursor Neoplasms

1. AML with Recurrent Genetic Abnormalities

AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*

AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*

Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12); *PML-RAR*

AML with t(9;11)(p22;q23); *MLLT3-MLL*

AML with t(6;9)(p23;q34); *DEK-NUP214*

AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*

AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*

AML with gene mutations

mutated *NPM1*

mutated *FLT3*

mutated *CEBPA*

mutated *KIT*

2. AML with Myelodysplastic-Related Changes

3. Therapy-Related Myeloid Neoplasms

4. AML, NOS

AML with minimal differentiation

AML without maturation

AML with maturation

Acute myelomonocytic leukemia

Acute monoblastic/monocytic leukemia

Acute erythroid leukemia (AEL)

Pure erythroid leukemia

Erythroleukemia, erythroid/myeloid

Acute megakaryoblastic leukemia

Acute basophilic leukemia

Acute panmyelosis with myelofibrosis

5. Myeloproliferations Related to Down Syndrome

Transient abnormal myelopoiesis (TAM)

Myeloid leukemia associated with Down syndrome

6. Blastic Plasmacytoid Dendritic Cell Neoplasm

7. Myeloid Sarcoma

III. Acute Leukemia of Ambiguous Lineage

Acute undifferentiated leukemia

Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR/ABL1*

Mixed phenotype acute leukemia with t(v;11q23); *MLL* rearranged

Mixed phenotype acute leukemia, B/myeloid, NOS

Mixed phenotype acute leukemia, T/myeloid, NOS

Provisional

Natural killer (NK) cell lymphoblastic leukemia/lymphoma

IV. Precursor Lymphoid Neoplasms

1. B Lymphoblastic Leukemia/Lymphoma

B lymphoblastic leukemia/lymphoma, NOS

B lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities

B lymphoblastic leukemia/lymphoma with t(9;22)(q34;q11.2); *BCR-ABL1*

B lymphoblastic leukemia/lymphoma with t(v;11q23); *MLL* rearranged

B lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22); *TEL-AML1* (*ETV6-RUNX1)*

B lymphoblastic leukemia/lymphoma with hyperdiploidy

B lymphoblastic leukemia/lymphoma with hypodiploidy (hypodiploid ALL)

B lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32); *IL3-IGH*

B lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1)*

2. T Lymphoblastic Leukemia/Lymphoma

V. Mature B-Cell Neoplasms

Chronic lymphocytic leukemia/small lymphocytic lymphoma

B-cell prolymphocytic leukemia

Splenic marginal zone lymphoma

Hairy cell leukemia

Splenic B-cell lymphoma/leukemia, unclassifiable

Lymphoplasmacytic lymphoma

Waldenström macroglobulinemia

Heavy chain diseases

α-heavy chain disease

γ-heavy chain disease

μ-heavy chain disease

Plasma cell neoplasms

Monoclonal gammopathy of undetermined significance (MGUS)

Plasma cell myeloma

Solitary plasmacytoma of bone

Extraosseous plasmacytoma

Monoclonal immunoglobulin deposition diseases

Osteosclerotic myeloma

Extranodal marginal zone lymphoma of mucosa-associate lymphoid tissue (MALT lymphoma)

Nodal marginal zone lymphoma

Pediatric nodal marginal zone lymphoma

Follicular lymphoma

Pediatric follicular lymphoma

Primary cutaneous follicle center lymphoma

Mantle cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) NOS

T-cell/histiocyte rich large B-cell lymphoma

Primary DLBCL of the CNS

Primary cutaneous DLBCL, leg type

EBV positive DLBCL of the elderly

DLBCL associated with chronic inflammation

Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK positive large B-cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Primary effusion lymphoma
Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma

VI. Mature T-Cell and NK Cell Neoplasms

T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK cells
Aggressive NK cell leukemia
Systemic EBV positive T-cell lymphoproliferative disease of childhood
Hydroa vacciniforme-like lymphoma
Adult T-cell leukemia/lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-associated T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous CD30+ T-cell lymphoproliferative disorders
  Lymphomatoid papulosis
  Primary cutaneous anaplastic large cell lymphoma
Primary cutaneous γ-δ-T-cell lymphoma
Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma
Primary cutaneous CD4+ small/medium T-cell lymphoma
Peripheral T-cell lymphoma, NOS
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma, *ALK* positive
Anaplastic large cell lymphoma, ALK negative

VII. Hodgkin Lymphoma

1. Nodular lymphocyte predominant
2. Classical
   Nodular sclerosis
   Lymphocyte rich
   Mixed cellularity
   Lymphocyte depleted

VIII. Histiocytic and Dendritic Cell Neoplasms

Histiocytic sarcoma
Langerhans cell histiocytosis
Langerhans cell sarcoma
Interdigitating dedritic cell sarcoma
Follicular dendritic cell sarcoma
Fibroblastic reticular cell tumor
Indeterminate dendritic cell tumor
Disseminated juvenile xanthogranuloma

IX. Post-Transplant Lymphoproliferative Disorders (PTLD)

Early lesions
  Plasmacytic hyperplasia
  Infectious mononucleosis-like PTLD
Polymorphic PTLD
Monomorphic PTLD (B- and T-/NK cell types)
Classical Hodgkin lymphoma type PTLD

Those entities in italics are provisional. At the time this classification was made, there was not enough evidence to recognize them as distinct entities.

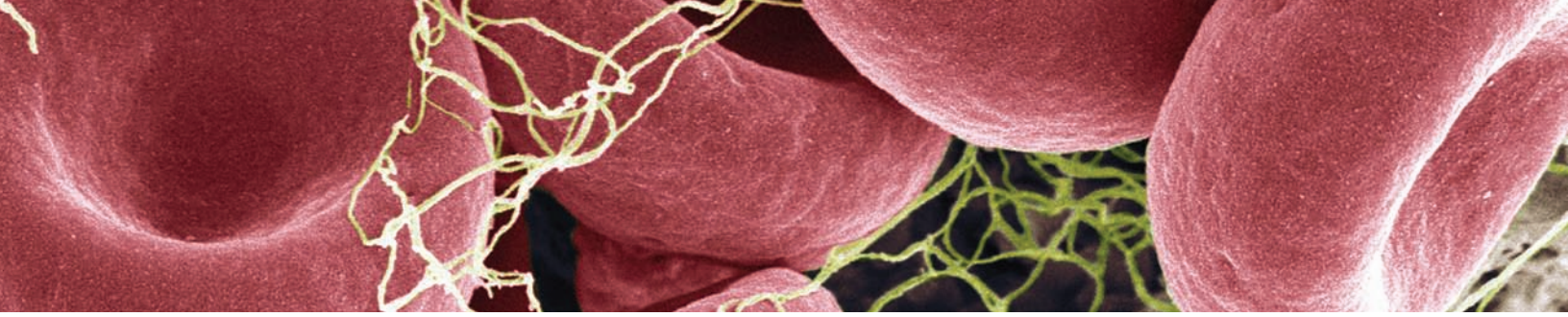
Adapted from: Swerdlow SH, Campo E, Harris NL et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC; 2008.

References

Vardiman JW, Thiele J, Arber DA et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–51.

Campo E, Swerdlow SH, Harris NL et al. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood*. 2011;117:5019–32.

Swerdlow SH, Campo E, Harris NL et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th ed. Lyon, France: IARC; 2008.



Glossary

Abetalipoproteinemia (hereditary acanthocytosis) rare, autosomal recessive disorder characterized by the absence of serum β-lipoprotein, low serum cholesterol, low triglyceride, and low phospholipid and an increase in the ratio of cholesterol to phospholipid.

Acanthocyte abnormally shaped erythrocyte with spicules of varying length irregularly distributed over the cell membrane's outer surface; has no central area of pallor. Also known as *spur cell.*

Achlorhydria absence of hydrochloric acid in stomach gastric secretions.

Acquired aberration chromosome abnormality (either numerical or structural) that occurs at some time after birth and involves only one cell line.

Acquired immune deficiency syndrome (AIDS) disease caused by infection with human immunodeficiency virus type I (HIV-1) that selectively infects helper T lymphocytes (CD4+), causing rapid depletion of these cells. This causes a deficiency in cell-mediated immunity. Patients have repeated infections with multiple opportunistic organisms and an increase in malignancies.

Acquired inhibitor *See* Circulating inhibitor (anticoagulant).

Acrocentric having a chromosome that has the centromere close to the terminal end so that the short arm is much shorter than the long arm. The short arm consists only of a stalk and a small amount of DNA called a *satellite.*

Acrocyanosis *See* Raynaud's phenomenon.

Activated partial thromboplastin time (APTT) screening test used to detect deficiencies in the intrinsic and common pathway of the coagulation cascade.

Activated lymphocyte *See* Reactive lymphocyte.

Activated protein C resistance (APCR) hemostatic condition in which activated protein C is not able to inactivate F-V, which could cause or contribute to thrombosis. In most cases, it is caused by a mutation in F-V in which Arg 506 is replaced with Gln (F-VLeiden).

Acute leukemia malignant hematopoietic stem cell disorder characterized by unregulated proliferation and block in maturation of a mutated stem cell or progenitor cell resulting in accumulation of immature and nonfunctional hematopoietic cells in the bone marrow, peripheral blood, and other organs.

Acute lymphocytic leukemia (ALL) malignant disorder characterized by unregulated proliferation and block in maturation of a mutated lymphoid progenitor cell resulting in accumulation of lymphoid cells in the bone marrow. Peripheral blood smear reveals the presence of many undifferentiated or minimally differentiated cells.

Acute myeloid leukemia (AML) malignant myeloproliferative disorder characterized by unregulated proliferation and a block in maturation of a mutated hematopoietic stem cell or myeloid progenitor cell resulting in accumulation of primarily undifferentiated or minimally differentiated myeloid cells in the bone marrow and peripheral blood.

Acute phase reactant plasma protein that rises rapidly in response to inflammation, infection, or tissue injury.

Acute undifferentiated leukemia (AUL) critical disorder in which the morphology, cytochemistry, and immunophenotype of the proliferating blasts lack sufficient information to classify them as myeloid or lymphoid origin.

ADAMTS-13 *See* **a** **d**isintegrin-like **a**nd **m**etalloprotease with **t**hrombo**s**pondin.

Adaptive immune response interaction of the T lymphocyte, B lymphocyte, and macrophage in a series of events that allows the body to attack and eliminate foreign antigens.

ADCC *See* Antibody-dependent cell cytotoxicity.

Adipocyte cell whose cytoplasm is largely replaced with a single fat vacuole; fat cell.

A disintegrin-like and metalloprotease with thrombospondin type motif (ADAMTS-13) metalloprotease enzyme responsible for cleavage of the ultralarge multimers of VWF released from endothelial cells into the VWF multimer sizes normally found in the circulation. Mutations in or deficiencies of this enzyme is a risk for thrombosis.

Adsorbed plasma platelet-poor fluid part of blood that is adsorbed with either barium sulfate or aluminum hydroxide to remove the coagulation factors II, VII, IX, and X (the prothrombin group). Factors V, VIII, XI, XII, and fibrinogen (I) are present in adsorbed fluid which is one of the reagents used in the substitution studies to determine a specific factor deficiency.

Advanced HIV disease stage of infection with the retrovirus HIV in which there are decreased CD4+ T lymphs and the presence of conditions that require antiretroviral therapy

Afibrinogenemia condition in which coagulation factor I, also called fibrinogen, is absent in the peripheral blood. It can be caused

by a mutation in the gene controlling the production of this plasma protein or by an acquired condition in which the plasma protein is pathologically converted to fibrin.

Aged serum protein-rich liquid portion of blood that lacks coagulation factors fibrinogen (I), prothrombin (II), V, VIII. It is prepared by incubating normal serum for 24 hours at 37°C. Factors VII, IX, X, XI, and XII are present in aged serum, which is one of the reagents used in substitution studies to determine a specific factor deficiency.

Agglutinate clumping of erythrocytes as a result of interactions between membrane antigens and specific antibodies.

Agglutination the process of erythrocyte clumping.

Aggregating reagent chemical substance (agonist) that promotes platelet activation and aggregation by attaching to a receptor on the platelet's surface.

Agonist chemical substance that can attach to a platelet membrane receptor and activate platelets, causing them to aggregate (e.g., collagen, ADP). It is used in the laboratory to test platelet function using a platelet aggregometer or platelet function analyzer.

α-granule (αG) *See* Alpha (α) granule.

Agranulocytosis absence of white blood cells in the peripheral blood.

AIDS-related complex (ARC) second recognized clinical stage of a person infected with the HIV virus. This category includes immune-compromised patients with mild symptoms of weight loss, fever, lymphadenopathy, thrush, chronic rash, or intermittent diarrhea.

Alder-Reilly anomaly benign condition characterized by the presence of functionally normal leukocytes with large purplish granules in their cytoplasm when stained with a Romanowsky stain.

Aleukemic leukemia disorder in which the abnormal malignant cells are found only in the bone marrow.

Allele one of two or more genes that correspond to the same trait and occupy the same position on paired chromosomes.

Alloantibody blood protein produced in one individual in response to the antigens of another individual of the same species.

Allogeneic pertaining to an allograft in which donor and host belong to the same species but are not genetically identical.

Allogeneic stem cell transplantation process of moving stem cells between genetically dissimilar animals of the same species.

Alloimmune hemolytic anemia hemolytic disorder generated when blood cells from one person are infused into a genetically unrelated person. The recipient's lymphocytes recognize antigens on the infused donor cells as foreign, stimulating the production of antibodies that react with donor cells and cause hemolysis.

Alpha (α) granule small particle of platelet storage containing a variety of proteins that are released into an area after platelet activation.

Analytical measurement range instrument's linearity field.

Analytical sensitivity ability to detect small amounts of an analyte.

Analytical specificity ability to detect only the analyte in question.

Analytical time period between entering a specimen into the test system and reporting the result by the instrument.

Anaplastic large cell lymphoma subtype of T/NK cell neoplasm characterized by large bizarre anaplastic cells that can resemble Reed-Sternberg and Hodgkin variant cells; cells are usually positive for the CD30 antigen but usually lack CD15 expression and evidence of Epstein-Barr virus infection; often positive for leukocyte common antigen (LCA) and epithelial membrane antigen (EMA).

Anemia disorder characterized by decrease in the normal concentration of hemoglobin or erythrocytes; can be caused by increased erythrocyte loss or decreased erythrocyte production and can result in hypoxia.

Anemia of chronic disease abnormal state caused by decrease in hemoglobin that accompanies many chronic conditions such as rheumatoid arthritis. It is characterized by low serum iron but normal iron stores. There is a block in iron release from macrophages for iron recycling mediated by hepcidin produced in response to IL-6, an inflammatory cytokine. Also called *anemia of inflammation.*

Anemia of inflammation other term for *anemia of chronic disease.*

Aneuploid used to describe a condition in which the number of chromosomes per cell does not equal a multiple of the haploid number, n; for example, in human cells, a chromosome count of 45, 47, 48, etc.

Anisocytosis general variation in erythrocyte size.

Anisoylated plasminogen streptokinase activator complex (APSAC) conglomerate of the enzyme streptokinase that is a chemically altered complex of streptokinase and plasminogen and is used as a thrombolytic agent in the treatment of thrombosis.

Annexin II endothelial cell receptor for tPA that functions as a coreceptor for both tPA and PLG. Binding and activation of tPA and PLG help maintain a fibrinolytic potential on undamaged vascular surfaces.

Antibody immunoglobulin produced in response to an antigenic substance.

Antibody-dependent cell cytotoxicity (ADCC) mechanism of the recognition and lysis of organisms by NK cells through binding IgG to the NK cell CD16 receptor. Any target organism coated with IgG can be bound to NK cells and lysed. Monocytes, macrophages, and neutrophils also have this receptor and act in a similar manner.

Anticardiolipin antibody (ACA) immunoglobulin produced by an organism in response to a constituent of its own tissues; is directed against negatively charged phospholipids. *See* Antiphospholipid antibody.

Anticoagulant chemical substance added to whole blood to prevent it from clotting. Depending on its type, in vitro clotting is prevented by the removal of calcium (EDTA) or the inhibition of the serine proteases such as thrombin (heparin).

Antigen any foreign substance that evokes antibody production (an immune response) and reacts specifically with that antibody.

Antigen-dependent lymphopoiesis development of immunocompetent lymphocytes into effector T and B lymphocytes that mediate the immune response through production of lymphokines and antibodies. The process, which occurs in secondary lymphoid

tissue, is initiated when mature lymphocytes come into contact with an antigen.

Antigen-independent lymphopoiesis development of lymphoid stem cells into immunocompetent T and B lymphocytes (virgin lymphocytes). This process occurs in the primary lymphoid tissue under the regulation of hematopoietic growth factors.

Antigen-presenting cell (APC) macrophage in the immune response; the macrophage phagocytizes substances foreign to the host and presents the foreign substance's antigenic determinants on its membrane to antigen-dependent T lymphocytes.

Antihuman globulin (AHG) protein used in a laboratory procedure designed to detect the presence of antibodies directed against erythrocyte antigens on the erythrocyte membrane.

Antioncogene unit of heredity that codes for a normal substance that suppresses tumor formation. Maturation and/or absence of both alleles allows tumor growth. Also called *tumor-suppressor gene.*

Antiphospholipid antibody immunoglobulin produced in response to an antigenic substance and directed against antigens that consist of a negatively charged phospholipid. Clinically important antiphospholipid immunoglobulins include anticardiolipin antibody (ACA) and lupus anticoagulant (LA). In some individuals, these immunoglobulins are associated with thrombosis and other hemostatic defects.

Antiphospholipid antibody syndrome clinical condition characterized by the presence of high titers of antiphospholipid immunoglobulin, thrombocytopenia, and recurrent arterial and venous thromboses, often affecting young males.

Aperture small opening with electrodes located on either size through which blood cells are drawn into an electronic cell counter. Electrical resistance is detected as the cell passes through the aperture.

Apheresis separation, removal, or withdrawal of whole blood from the donor or patient and separated into its components, one of which is retained; the remaining constituents are recombined and returned to the individual.

Aplasia failure of hematopoietic cells to generate and develop in the bone marrow.

Aplastic anemia disorder characterized by peripheral blood pancytopenia and hypoplastic marrow. It is considered a pluripotential stem cell disorder.

Aplastic crisis abrupt, transient cessation of erythropoiesis that occurs in some hemolytic anemias and infections.

Apoferritin cellular protein that combines with iron to form ferritin; found attached only to iron, not in the free form.

Apotransferrin iron transport protein without iron attached. When the protein has iron attached, it is referred to as *transferrin*.

Apoptosis programmed cell death resulting from activation of a predetermined sequence of intracellular events; "cell suicide."

APSAC *See* Anisoylated plasminogenstreptokinase activator complex.

APTT *See* Activated partial thromboplastin time.

Arachidonic acid (AA) unsaturated essential fatty acid, usually attached to the second carbon of the glycerol backbone of phospholipids, released by phospholipase A_2; a precursor of prostaglandins and thromboxanes.

Arachnoid mater delicate membrane that covers the central nervous system; middle layer of the meninges.

Artificial oxygen carrier (AOC) a class of manufactured blood that has two groups including hemoglobin-based oxygen carriers (HBOCs) in solution and perfluorocarbons (PFCs). The HBOCs consist of purified human or bovine hemoglobin and recombinant hemoglobin. The oxygen dissociation curve of HBOCs is similar to that of native human blood. Hemoglobin tests based on colorimetric analysis could give erroneous results. PFCs are fluorinated hydrocarbons with high gas-dissolving capacity. They do not mix in aqueous solution and must be emulsified. In contrast to HBOCs, a linear relationship exists between PO_2 and oxygen content in PFCs. Thus, relatively high O_2 partial pressure is required to maximize delivery of O_2 by PFCs.

Ascite effusion and accumulation of fluid in the peritoneal cavity.

Ascitic fluid liquid substance that abnormally collects in the peritoneal cavity of the abdomen.

Atypical chronic myeloid leukemia (aCML, *BCR/ABL1*$^-$) variant condition of MDS/MPN characterized by primary involvement of the neutrophil series with leukocytosis involving dysplastic immature and mature neutrophils. Multilineage dysplasia is common. Does not have the *BCR/ABL1* gene mutation.

Atypical lymphocyte *See* Reactive lymphocyte.

Auer rod reddish-blue staining needlelike inclusion within the cytoplasm of leukemic myeloblasts that occur as a result of abnormal cytoplasmic granule formation. Their presence on a Romanowsky-stained smear is helpful in differentiating acute myeloid leukemia from acute lymphoblastic leukemia.

Autoantibody immunoglobulin produced in response to an antigenic substance in the blood capable of reacting with the subject's own antigens.

Autocrine *See* Autocrine signaling.

Autocrine signaling process that produces a signal that acts on the same cell that produced it.

Autohemolysis process of destruction of the subject's own erythrocytes by hemolytic agents in the subject's blood.

Autoimmune hemolytic anemia (AIHA) condition that results when individuals produce antibodies against their own erythrocytes. The antibodies are usually against high-incidence antigens.

Autologous derived from self.

Autologous stem cell transplantation infusion of a person's own stem cells.

Autosome chromosome that does not contain genes for sex differentiation; in humans, chromosome pairs 1–22.

Autosplenectomy extensive splenic damage secondary to infarction; often seen in older children and adults with sickle cell anemia.

Azurophilic granule small particle (primary) within myelocytic leukocytes that has a predilection for the aniline component of a Romanowsky-type stain. This particle appears bluish purple or bluish black when observed microscopically on a stained blood smear. It first appears in the promyelocyte.

Backlighting highlighting a parameter that falls outside its reference interval or a user-defined action limit; alerts the clinical laboratory professional to a potential problem or error that requires further investigation.

Band neutrophil cell type that is immediate precursor of the mature granulocyte. It can be found in either the bone marrow or peripheral blood. The nucleus is elongated, and nuclear chromatin is condensed. The cytoplasm stains pink, and there are many specific granules. The cell is 9–15 mcM (μm) in diameter. Also called *stab* or *unsegmented neutrophil.*

Barr body (drumstick) chromatin body; inactive X chromosome that appears as an appendage of the neutrophil nucleus but are not visible in every neutrophil.

Basophil mature granulocytic cell characterized by the presence of large readily stained granules that are purple blue or purple black with Romanowsky stain. The cell is 10–14 mcM (μm) in diameter, and the nucleus is segmented. Granules are cytochemically positive with periodic acid-Schiff (PAS) and peroxidase and contain histamine and heparin peroxidase. This granulocytic cell constitutes $<0.2 \times 10^9$/L or 0–1% of peripheral blood leukocytes, functions as a mediator of inflammatory responses, and has receptors for IgE.

Basophilia increased concentration of circulating basophils.

Basophilic normoblast nucleated precursor of the erythrocyte that is derived from a pronormoblast. The cell is 10–16 mcM (μm) in diameter. The nuclear chromatin is coarser than the pronormoblast, and nucleoli are usually absent. Cytoplasm is more abundant, and it stains deeply. The cell matures to a polychromatophilic normoblast. Also called *prorubricyte.*

Basophilic stippling presence of precipitating ribonucleoproteins and mitochondrial remnants that compose erythrocyte inclusions. Observed on Romanowsky-stained blood smears as diffuse or punctate bluish-black granules in toxic states such as drug (lead) exposure. Diffuse, fine basophilic stippling can occur as an artifact.

B-cell acute lymphoblastic leukemia (B-ALL) immunologic type of cancer of the blood and bone marrow in which the neoplastic cell is a B lymphoid cell; it has subtypes.

B-cell receptor (BCR) specific entity on the B lymphocyte membrane that reacts with antigen.

***bcl-2* gene** hereditary unit on chromosome 18 producing bcl-2 protein. The translocation t(14;18) found in follicular lymphoma leads to bcl-2 overexpression and inhibition of lymphocyte cell death.

Beer-Lambert's law formula for the mathematical basis for colorimetry. The equation is $A = C \times L \times K$. A is absorbance, C is the concentration of the colored substance, L is the depth of the solution through which the light travels, and K is a constant.

Bence-Jones proteinuria excessive immunoglobulin light chain found in the urine from patients with multiple myeloma.

Benign nonmalignant tissue formed from highly organized, differentiated cells that do not spread or invade surrounding tissue.

Bernard-Soulier syndrome rare autosomal-recessive hereditary platelet disorder characterized by a moderate to severe thrombocytopenia, giant platelets, and abnormal platelet function. The defect is a quantitative decrease or abnormal function of the GPIb/IX/V complex resulting in the platelets' inability to adhere to collagen.

BFU-E *See* Burst-forming unit–erythroid.

Bilineage acute leukemia malignant blood cell condition that is characterized by the presence of two separate populations of malignant cells, one of which phenotypes as lymphoid and the other as myeloid.

Bilirubin breakdown product of the heme portion of the hemoglobin molecule. Initial steps in the degradation of hemoglobin result in a lipid-soluble form (unconjugated or indirect bilirubin) that travels in the blood stream to the liver. There it is converted into a water-soluble form (conjugated or direct bilirubin) that can be excreted into the bile.

Bioavailability degree and rate at which a free drug is available to produce its effect.

Biphasic antibody immunoglobulin that binds to erythrocytes at room temperature or below and causes hemolysis when the blood warms to 37°C.

Biphenotypic acute leukemia malignant blood cell disorder in which there is an unregulated proliferation and block in maturation of mutated progenitor cells that have myeloid and lymphoid markers on the same population of neoplastic cells.

Birefringence quality of a substance that can change the direction of light rays that are directed at a substance; can be used to identify crystals.

2,3-bisphosphoglycerate (2,3-BPG) product of the glycolytic pathway that affects the oxygen affinity of hemoglobin. It serves in the biochemical feedback system that regulates the amount of oxygen released to the tissues. As the concentration of 2,3-BPG increases, hemoglobin's affinity for oxygen decreases and more oxygen is released to the tissue. Also referred to as *2,3-diphosphoglycerate* (*2,3-DPG*).

Bite cell erythrocyte with a portion of the cell missing; seen in G6PD deficiency and drug-induced oxidant hemolysis.

Blast crisis evolution of a chronic hematopoietic neoplasm to an acute leukemia. Various genetic changes in the leukemic stem cells accompany this terminal stage of the disease.

Blast transformation complex cell process that occurs after contact and binding of an antigen to the antigen receptors of T- and B-immunocompetent lymphocytes; result is the clonal amplification of cells responsible for the expression of immunity to that specific antigen.

Bleeding time and PFA 100 screening test that measures platelet function.

Blinded preanalyzed sample previously tested specimen that is integrated randomly into a specimen run and possesses no identifying feature (e.g., number or designation) to indicate that it differs from current patient specimens. Only the individual who selected and relabeled it as part of a quality control program can identify them.

Blister cell cell with a clear area next to the membrane on one side; thought to be formed when the phagocyte removes a Heinz body in the cell and is seen in G6PD deficiency.

Blood coagulation formation of a blood clot, usually considered a normal process.

Blood island cluster of cells in the yolk sac of the human embryo that gives rise to yolk sac erythroblasts.

B lymphoid cell antigen CD marker that when present is unique to and useful in differentiating a B-cell ALL from other types of acute leukemia.

Bohr effect result of pH on hemoglobin-oxygen affinity; serves as one of the most important buffer systems in the body. As the H^+ concentration in tissues increases and the affinity of hemoglobin for oxygen decreases, oxygen unloading is permitted.

Bone marrow aspirate fluid withdrawn from the bone marrow by aspiration using a special needle (e.g., Jamshidi needle) and syringe. The fluid represents the specialized soft tissue that fills the medullary cavities between the bone trabeculae. Its examination is useful in evaluating hematopoietic cellular morphology, distribution, and development; observing for presence of abnormal cells; and estimating cellularity.

Bone marrow trephine biopsy removal of a small piece of a soft highly vascular modified connective tissue within the core of bone that contains hematopoietic tissue, fat, and trabcula. Examination of the trephine biopsy is useful in observing the tissue's architecture and cellularity and allows interpretation of the spatial relationships of bone, fat, and marrow cellularity.

Bordetella pertussis gram-negative aerobic coccobacilli that causes whooping cough. The hematologic picture in whooping cough is leukocytosis with lymphocytosis. The lymphocytes are small cells with folded nuclei.

Bronchoalveolar lavage sample of fluid obtained by a diagnostic procedure of infusing and removing a sterile saline solution into the alveolar and bronchial airspaces of the lung via a bronchoscope. It is performed to detect diseases of the lower respiratory tract.

Buffy coat layer of white blood cells and platelets that lies between the plasma and erythrocytes in centrifuged blood sample.

Burkitt cell lymphoblast that is found in Burkitt's lymphoma.

Burst-forming unit erythroid Committed red blood cell progenitor that develops into the unipotential CFU-E stem cell. It is relatively insensitive to EPO except in high concentrations. GM-CSF stimulates it to enter the cell cycle.

Butt cell circulating neoplastic lymphocyte with a deep indentation (cleft) of the nuclear membrane. Butt cells can be seen when follicular lymphoma involves the peripheral blood.

Cabot ring reddish-violet erythrocyte inclusion resembling the figure 8 on Romanowsky-stained blood smears that can be found in some cases of severe anemia.

Cancer-initiating cell cell from which cancer originally emerges; sometimes called the *cell of origin*, it has the capacity for unlimited self-renewal.

Cancer stem cell cell within a tumor that is capable of extensive proliferation in vitro (in colony-forming assays) and in vivo (in transplantation models). It is believed to be rare within a tumor but has infinite proliferative potential that drives the formation and growth of tumors.

Capitated payment reimbursement method for health care by third-party payers in which the insurer contracts with certain health care providers who agree to provide services for a defined population on a per-member fee schedule. The insurer determines who the providers will be.

Carboxyhemoglobin compound formed when hemoglobin is exposed to carbon monoxide; it is incapable of oxygen transport.

Carboxylation addition of a carboxyl group to coagulation factors in the prothrombin group (II, VII, IX, X, proteins C, S, Z) in the liver; it is necessary for the factor to become functional. Vitamin K is required for this reaction.

Cardiac tamponade critical clinical condition in which the pericardial sac fills with fluid and restricts the heartbeat and venous return to the heart.

Caspase cysteine protease responsible for cell alterations in apoptosis.

Catalytic domain area in a protein that is common to all serine proteases involved in blood clotting. Cleavage of a peptide bond occurs here and converts the proenzyme to its active form.

CD designation name of a cluster of differentiation of a group of monoclonal antibodies recognizing the same protein marker antigen on a cell. The antibodies are used to classify cell types and stages of maturation.

Cell cycle biochemical and morphological stages a cell passes through leading up to its division; includes G1, S, G2, and M phases.

Cell cycle checkpoint place in the cell cycle at which its progress can be halted until conditions are suitable for the cell to proceed to the next stage.

Cell-mediated immunity resistant response mediated by T lymphocytes that requires interaction between histocompatible T lymphocytes and macrophages with antigen. At least three important T lymphocyte subsets are involved: helper, regulatory and cytotoxic.

Cellular hemoglobin concentration mean (CHCM) erythrocyte index that represents the average hemoglobin concentration of individual cells analyzed. It is derived from the hemoglobin histogram. Interference with the hemoglobin determination resulting from turbidity or lipemia can be identified by comparing it to the MCHC.

Central nervous system (CNS) part of the nervous system that consists of the brain and spinal cord.

Centriole cytoplasmic organelle that is the point of origin for the contractile protein known as *spindle fiber.*

Centromere primary constriction that attaches sister chromatids in a chromosome, dividing them into long and short arms.

Cerebrospinal fluid (CSF) liquid substance normally produced to protect the brain and spinal cord. Produced by the choroid plexus cells and absorbed by the arachnoid pia, it circulates in the subarachnoid space.

Ceruloplasmin ferroxidase that converts Fe^{++} to Fe^{+++} for binding to transferrin. Export of iron from nonintestinal cells, including macrophages, requires this protein.

CFU-E *See* Colony-forming unit-erythroid.

CH50 functional hemolytic titration assay to measure lysis, the endpoint of complement activation. It measures the amount of patient serum required to lyse 50% of a standardized concentration of antibody-sensitized sheep red blood cells. Because all complement proteins are required for lysis to occur, any single complement factor deficiency causes a negative reaction (no lysis).

Charcot–Leyden crystal material formed from eosinophil granules that is found in tissues with large numbers of eosinophils.

Cheidak–Higashi anomaly multisystem disorder inherited in an autosomal recessive fashion and characterized by recurrent infections, hepatosplenomegaly, partial albinism, and central nervous system (CNS) abnormalities; neutrophil chemotaxis and killing of organisms is impaired. Giant cytoplasmic granular inclusions are in leukocytes and platelets.

Chemokine cytokine with chemotactic activity.

Chemotaxin chemical messenger that causes migration of cells in one direction. Also called *chemokine*.

Chemotaxis migration in response to a chemical signal or stimulation.

Chimerism state of being when cells from two different zygotes are expressed in one individual.

Chloride shift phenomenon in which a plasma chlorine ion diffuses into the erythrocyte when a free bicarbonate ion diffuses from the erythrocyte into the plasma.

Cholecystitis inflammation of the gallbladder.

Cholelithiasis formation of calculi or bile stones in the gallbladder or bile duct.

CHr reticulocyte hemoglobin content provided by Advia 120 and 2120 System by Bayer Diagnostics; analogous to the MCH index of erythrocytes.

Chromatid structure of DNA during G_0 and G_1 of the cell cycle. After S phase, DNA has been replicated, and the chromosome consists of two parallel, identical chromatids held together at the centromere.

Chromogenic assay spectrophotometric measurement of an enzyme's activity based on the release of a colored pigment following enzymatic cleavage of the pigment-producing substrate (chromogen).

Chromosome nuclear structure seen during mitosis and meiosis consisting of supercoiled DNA with histone and nonhistone proteins; consists of two identical (sister) chromatids attached at the centromere.

Chronic basophilic leukemia rare myeloproliferative neoplasm (MPN). It is characterized by an extreme increase in basophils in the peripheral blood. The cell of origin is the common myeloid progenitor cell or the CFU-Baso, a bipotential progenitor cell capable of differentiating into either basophil or mast cell lineages.

Chronic eosinophilic leukemia, not otherwise specified Clonal myeloproliferative neoplasm that presents with eosinophilia $\geq 1.5 \times 10^9$/L in blood that is not classified as another neoplastic condition and does not have the *PDGFRA, PDGFRB,* or *FGFR1* mutations.

Chronic idiopathic thrombocytopenic purpura (ITP) immune form of thrombocytopenia that occurs most often in young adults and lasts longer than 6 months.

Chronic lymphocytic leukemia (CLL) disorder characterized by a neoplastic growth of lymphoid cells in the bone marrow and an extreme elevation of these cells in the peripheral blood. It is characterized by leukocytosis, <20% blasts, and a predominance of mature lymphoid cells; classified by WHO as a mature B-lymphoid neoplasm.

Chronic idiopathic myelofibrosis (CIMF) myeloproliferative neoplasm characterized by excessive proliferation of all cell lines as well as progressive bone marrow fibrosis and blood cell production at sites other than the bone marrow, such as the liver and spleen. Also called *agnogenic myeloid metaplasia, myelofibrosis with myeloid metaplasia, and primary myelofibrosis.*

Chronic myelogenous leukemia (CML) neoplasm characterized by a neoplastic growth of primarily myeloid cells in the bone marrow and an extreme elevation of these cells in the peripheral blood. The two phases of the disease are chronic and blast crisis. The chronic phase has <20% blasts in the bone marrow or peripheral blood, whereas the blast crisis phase has >20% blasts. Individuals with this disease have the *BCR/ABL1* translocation, which codes for a unique P210 protein. Also referred to as *chronic granulocytic leukemia* (*CGL*).

Chronic myelomonocytic leukemia (CMML) subgroup of the myelodysplastic syndromes characterized by anemia and a variable total leukocyte count. An absolute monocytosis ($>1 \times 10^9$/L) is present; immature erythrocytes and granulocytes can be present. There are <5% blasts in the peripheral blood. The bone marrow is hypercellular with proliferation of abnormal myelocytes, promonocytes, and monoblasts, and there are <20% blasts.

Chronic neutrophilic leukemia myeloproliferative neoplasm (MPN) characterized by a sustained increase in neutrophils in the peripheral blood with a slight shift to the left. The Ph chromosome and *BCR/ABL1* translocation are absent.

Chronic nonspherocytic hemolytic anemia group of chronic conditions marked by a deficiency of red blood cells or of hemoglobin in the blood and premature erythrocyte destruction. Spherocytes are not readily found, which is helpful when differentiating these conditions from hereditary spherocytosis.

Chylous body effusion that has a milky, opaque appearance from the presence of lymph fluid and chylomicrons.

Circulating inhibitor (anticoagulant) acquired pathologic protein, primarily immunoglobulins (IgG or IgM) with antibody specificity toward a factor involved in fibrin formation. Circulating inhibitors that interfere with the factor's activity are associated with a number of conditions, such as hemophilia, autoimmune diseases, malignancies, certain drugs, and viral infections.

Circulating leukocyte pool population of neutrophils actively circulating within the peripheral blood stream.

Circulating pool *See* Circulating leukocyte pool.

Clinical and Laboratory Standards Institute (CLSI) volunteer-driven organization that promotes the development and use of

voluntary laboratory standards and guidelines; its mission is "to develop best practices in clinical and laboratory testing and promote their use throughout the world using a consensus-driven process that balances the viewpoints of industry, government, and the healthcare professions." (http://www.clsi.org/Content/NavigationMenu/AboutCLSI/VisionMissionandValues/Vision_Mission_Value.htm).

Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) regulations that mandate standards in clinical laboratory operations and testing signed into federal law in 1988.

Clonal hypereosinophilia progenitor cell disorder of eosinophils that is classified as either myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA, PDGFRB,* or *FGFR1* or as chronic eosinophilic leukemia, not otherwise specified (CEL-NOS).

Clonality presence of identical cells derived from a single progenitor. It can be detected by identifying only one of the immunoglobulin light chains (κ or λ) on B cells or the presence of a population of cells with a common phenotype.

Clonogenic ability of a cell to form a clone.

Clot extravascular coagulation whether occurring in vitro or in blood shed into the tissues or body cavities.

Clot retraction cohesion of a fibrin clot that requires adequate, functionally normal platelets. Retraction of the clot occurs over a period of time and results in the expression of serum and a firm mass of cells and fibrin.

Cluster analysis method of classifying floating thresholds of large group of specific cell populations based on size and staining or absorption characteristics. It uses an instrument that can accommodate for shifts in abnormal cell populations from one sample to another sample.

CLSI *See* Clinical and Laboratory Standards Institute.

Coagulation factor soluble inert plasma protein that interacts to form fibrin after an injury.

Cobalamin cobalt-containing complex that is common to all subgroups of the vitamin B_{12} group.

Codocyte *See* Target cell.

Codon sequence of three nucleotides that encodes a particular amino acid.

Coefficient of determination (r^2) statistic that represents the square of the correlation coefficient. It is a measure of the strength of the relationship between two data sets.

Coefficient of variation relative standard deviation or standard deviation expressed as a percentage of the mean for a set of data.

Cofactor coagulation component that functions together with other coagulation factors. It is required for the conversion of specific zymogens to the active enzyme form.

Coincidence in an electronic cell counter, a phenomenon when two or more cells cross the sensing zone at the same time and are evaluated as only one cell.

Cold agglutinin disease *See* Cold agglutinin syndrome.

Cold agglutinin syndrome condition associated with the presence of cold-reacting autoantibodies (IgM) directed against erythrocyte surface antigens. This causes clumping of the red cells at room or lower temperatures.

Colony-forming unit visible aggregation (in vitro) of cells that developed from a single stem cell.

Colony-forming unit-erythroid (CFU-E) unipotential stem cell derived from the BFU-E. It has a high concentration of EPO membrane receptors and with EPO stimulation transforms into a pronormoblast, the earliest recognizable erythroid precursor.

Colony-stimulating factor cytokine that stimulates the growth of immature leukocytes in the bone marrow.

Column chromatography laboratory separation method based on the differential distribution of a liquid or gaseous sample (mobile phase) that flows through a sequence of specific substance (stationary phase). Depending on the chemical characteristics of the stationary phase, the substance of interest can bind to the stationary phase and remain in the column or directly pass through the column and remain in the mobile phase. If the substance remains in the column, a second mobile phase (elution buffer) is used to release the substance from the stationary phase and allow it to pass through the column.

Commitment state of two cells derived from the same precursor cell each of which takes a separate route of development.

Committed/progenitor cell parent or ancestor organism that differentiates into one cell line.

Common coagulation pathway one of the three interacting pathways in the coagulation cascade. The common pathway includes three rate-limiting steps: (1) activation of factor X by the intrinsic and extrinsic pathways, (2) conversion of prothrombin to thrombin by activated factor X, and (3) cleavage of fibrinogen to fibrin.

Comparative genomic hybridization (CGH) assay that can be used to analyze changes in chromosome copy number (copy number variants) and critical regions of the DNA for well-defined genetic abnormalities.

Compensated hemolytic disease disorder in which the erythrocyte life span is decreased but the bone marrow is able to increase erythropoiesis enough to compensate for the decrease; anemia does not develop.

Compensation process of adjusting the settings on the flow cytometer or performing a mathematical correction for overlap of light emitted by several fluorochromes either before or after the data are collected.

Competency assessment mechanism for determining the requisite ability that personnel have to perform a given laboratory procedure. It includes recognition of specimen collection errors, interpretation of test results to detect possible instrument or specimen problems and of quality control results, investigation of instrument or specimen problems, and proper reporting of results.

Complement any one of the 11 serum proteins that causes lysis of the cell membrane when sequentially activated.

Complementary DNA synthetic DNA transcribed from an RNA template by the enzyme reverse transcriptase. Also known as *cDNA*.

Complete blood count (CBC) hematology screening test that includes the white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin, hematocrit, and, often, platelet count. It can also include red cell indices.

Compression syndrome condition of altered physiological function of an organ or tissue because of impingement by an abnormal mass.

Compound heterozygote individual possessing two different abnormal alleles of a gene.

Conditioning regimen a treatment process in which high-dose chemotherapy and/or irradiation given to a patient before stem cell transplantation.

Congenital present at birth.

Congenital aberration chromosome abnormality (either numerical or structural) that is present at the time of birth in all cell lines or in several cell lines in the case of mosaicism.

Congenital amegakaryocytic thrombocytopenia (CAMT) condition present at birth with decreased marrow megakaryocytes and decreased platelets in the peripheral blood which eventually converts into bone marrow failure and aplastic anemia. Most cases are caused by mutations of the gene for the thrombopoietin receptor (c-*mpl*).

Congenital Heinz body hemolytic anemia inherited disorder characterized by anemia resulting from decreased erythrocyte life span. Erythrocyte hemolysis results from the precipitation of hemoglobin in the form of Heinz bodies, which damage the cell membrane and cause cell rigidity.

Congenital thrombocytopenia with radioulnar synostosis (CTRUS) disorder present at birth that presents with decreased marrow megakaryocytes and decreased platelets in the peripheral blood, which eventually converts into bone marrow failure and aplastic anemia. Most cases are caused by mutations within the *HOXA11* gene (which codes for a regulatory protein involved in the development of hematopoietic and bone tissue).

Consolidation therapy second phase of cancer chemotherapy whose function is to damage or kill those malignant cells that were not destroyed during the induction phase.

Constitutional aberration genetic abnormality present in every cell in a patient's body.

Constitutional cytogenetic aberration genetic abnormality found in every cell of the body.

Consumption coagulopathy *See* Disseminated intravascular coagulation.

Contact group coagulation factors in the intrinsic pathway involved with the initial activation of the coagulation system including factors XII, XI, prekallikrein, and high-molecular-weight kininogen. Activation of the factors requires contact with a negatively charged surface.

Continuous flow analysis automated method of analyzing blood cells that allows measurement of cellular characteristics such as individual cells that flow singly through a laser beam.

Contour gating subclassification of cell populations based on two characteristics such as size (x-axis) and nuclear density (y-axis) and the frequency (z-axis) of that characterized cell type. This information is used to create a three-dimensional plot and to draw a line along the valley between two peaks to separate the two cell populations.

Correlation coefficient (r) multiplier or factor that measures the distribution of data about the estimated linear regression line.

Coverglass smear a thin film of blood prepared by placing a drop of blood in the center of one cover glass and then placing a second cover glass on top of the blood at a 45° angle to the first cover glass. The two cover glasses are pulled apart, creating two cover glass smears.

Critical limit critical high and low value for a test result. Values above or below these values pose a life-threatening situation and are termed *panic values*.

Critical value laboratory result that reflects a life-threatening situation determined by a physician and for which an intervention is possible.

Crossover reciprocal exchange of genetic material between chromatids; normally occurs in meiosis to increase the diversity of the species.

Cross-reacting material negative (CRM−) description of defective clotting factor that can be identified by both abnormal functional and immunologic tests.

Cross-reacting material positive (CRM+) description of functionally defective clotting factor that can be identified by immunologic means.

Cross-reacting material reduced (CRM^R) description of defective clotting factor that is identified by an equal decline of both functional and immunological assays.

Cryoprecipitate preparation of proteins containing fibrinogen, von Willebrand factor, and factor VIII prepared by freezing and thawing plasma; used for replacement therapy in patients with hemophilia A and von Willebrand disease.

Cryopreserved preserved cell stored at very low temperatures while maintaining cell viability.

Cryosupernatant product that lacks large VWF multimers that are present in fresh frozen plasma yet still contains the VWF-cleaving protease missing in thrombotic thrombocytopenic purpura (TTP) patients.

Culling the spleen's filtration and destruction of senescent/damaged red cells.

Current Procedure Terminology (CPT) coding system in which numbers are assigned to laboratory tests (as well as medical, surgical, and other diagnostic services) by the American Medical Association's CPT Editorial Panel. Used for billing and record keeping.

Cyanosis bluish color of the skin and mucous membranes that develops as a result of excess deoxygenated hemoglobin in the blood.

Cyclin/Cdk kinase protein that regulates the transition between the various phases of the cell cycle.

Cytochemistry chemical staining procedure used to identify various constituents (enzymes and proteins) within white blood cells. It is useful in differentiating blasts in acute leukemia, especially

when morphologic differentiation on Romanowsky-stained smears is impossible.

Cytogenetic remission absence of recognized cytogenetic abnormalities associated with a given neoplastic disease (previously identified in a patient) after therapy.

Cytogenomic microarray analysis molecular genetic assessment technique that can be used to examine gene composition.

Cytokine protein produced by many cell types; it modulates the function of other cell types. The group includes interleukins, colony-stimulating factors, and interferons.

Cytomegalovirus (CMV) herpes virus that replicates only in human cells; has a widespread distribution and is spread by close contact with an infected person.

Cytoplasm protoplasm of a cell outside the nucleus.

Cytotoxic T cell (Cytotoxic T lymphocyte) effector cell produced from T immunoblasts.

DcytB duodenal cytochrome–B reductase; ferric reductase that decreases ferric iron to the ferrous state at the enterocyte brush border.

D-dimer cross-linked fibrin degradation product that is the result of plasmin's proteolytic activity on a fibrin clot. The presence of D-dimers is specific for fibrinolysis.

Decay-accelerating factor regulating complement protein found on cell membranes that accelerates decay (dissociation) of membrane-bound complement (C3bBb). Its absence leads to excessive sensitivity of these cells to complement lysis.

Deep vein thrombosis (DVT) formation of a blood clot in the deep veins (usually a leg vein).

Degranulation process in phagocytosis by which the neutrophil granules fuse with the phagosome and release their contents.

Dehydrated hereditary stomatocytosis (DHS) horizontal red blood cell membrane defect in which the cell is abnormally permeable to cations; net loss of intracellular K^+ exceeds the passive Na^+ influx, and net intracellular cation and water content are thus decreased; the cell dehydrates and appear targeted, contracted, and spiculated. Also known as *hereditary xerocytosis*.

Delayed bleeding symptom of severe coagulation factor disorders in which a wound bleeds a second time after initial stoppage. It occurs because the primary hemostatic plug is not adequately stabilized by the formation of fibrin.

Delta check comparison of current hematology results to the most recently reported previous result for a given patient; helps detect certain random errors.

Delta (δ) storage pool disease autosomal dominant disorder characterized by a decrease in dense granules in the platelets.

Demarcation membrane system cytoplasmic membrane system in the megakaryocyte that separates small areas of the cell's cytoplasm that eventually become a platelets.

Demargination movement of neutrophils that are loosely attached to endothelial cells lining the blood vessels into the circulation; can cause a pseudoneutrophilia.

Demyelination destruction, removal, or loss of the lipid substance that forms a myelin sheath around the axons of nerve fibers; a characteristic finding in vitamin B_{12} deficiency.

Dense granule small particle in platelet that stores nonmetabolic ADP, calcium, and serotonin as well as other compounds released from activated platelets.

Dense tubular system (DTS) complex made up of membranes in the platelet that originate from the smooth endoplasmic reticulum of the megakaryocyte. It is one of the storage sites for calcium ions within platelets, and its channels do not connect with the platelet's surface.

Densitometer instrument that can be used to measure the density of materials using different wavelengths of light and filters.

Densitometry laboratory testing method that determines the pattern and concentration of protein fractions separated by electrophoresis by measuring the amount of light absorbed by each dye-bound protein fraction as it passes a slit through which light is transmitted. The amount of light absorbed (optical density) is directly proportional to the protein's concentration.

Deoxyhemoglobin hemoglobin without oxygen.

Diagnosis Related Group (DRG) code system of about 500 medical conditions developed for Medicare as a part of the prospective payment system for inpatients. Reimbursement for care of hospitalized patients by almost all payers is not for each test or procedure but for the overall diagnosis. It is assigned when the patient is discharged and is severity adjusted (MS-DRG).

Diamond-Blackfan anemia congenital, progressive erythrocyte hypoplasia that occurs in very young children. There is no leukopenia or thrombocytopenia.

Diapedese passage of blood cells through the unruptured capillary wall. For leukocytes, this involves active locomotion.

Diapedesis *See* Diapedese.

Differentiation discernment of the appearance of various properties in cells that were initially equivalent.

2,3-diphosphoglycerate (2,3-DPG) See *2,3-bisphosphoglycerate* (*2,3-BPG*).

Dilute Russell viper venom time (dRVVT) test recommended as the screening test for detection of lupus anticoagulant; Russell viper venom (RVV) activates FX, resulting in clot formation. If LAs/aPLs are present, the patient's dRVVT is longer than that of the normal control.

Diploid number of chromosomes in somatic cells that are 2n. For human cells, $2n = 46$.

Direct antiglobulin test (DAT) laboratory test used to detect the presence of antibody and/or complement that is attached to the erythrocyte. It uses antibody directed against human immunoglobulin and/or complement. Also called the *antihuman globulin (AHG) test*.

Disseminated intravascular coagulation (DIC) complex condition in which the normal clotting process is altered (resulting in systemic rather than localized activation) by an underlying condition. Resulting complications can include thrombotic occlusion

of vessels, bleeding, and ultimately organ failure. DIC is initiated by multiple triggers, most involving damage to the endothelial lining of vessels.

DMT1 integral membrane protein that transports ferrous iron across the apical enterocyte plasma membrane.

DNA (deoxyribonucleic acid) blueprint that cells use to catalog, express, and propagate information; the fundamental substance of heredity that is carried from one generation to the next. It is a double-stranded molecule composed of complementary nucleotide sequences. The two strands of the substance are held together by hydrogen bonds formed according to the following rules of complementary nucleotide pairing: G bonds with C; A bonds with T; other combinations cannot bond.

DNA index (DI) DNA content of tumor cells relative to a diploid population of cells. It is calculated as the DNA content of cells in the tumor in the G_0/G_1 phase of the cell cycle relative to the DNA content of G_0/G_1 cells in a diploid control.

DNA sequencing Determining the nucleotide string in a segment of the blueprint that cells use to catalog, express, and propagate information by replicating its strands and monitoring the order in which labeled nucleotides are added to the new strands.

Double heterozygous having two different mutated genes that result in combination disorders. In the hemoglobinopathies, the most common structural hemoglobins inherited with thalassemia are HbC, HbS, HbE.

Döhle body oval aggregate of rough endoplasmic reticulum that stains light gray blue (with Romanowsky stain) found within the cytoplasm of neutrophils and eosinophils; associated with severe bacterial infection, pregnancy, burns, cancer, aplastic anemia, and toxic states.

Donath-Landsteiner antibody biphasic IgG associated with paroxysmal cold hemoglobinuria; reacts with erythrocytes in capillaries at temperatures below 15°C and fixes complement to the cell membrane. Upon warming, the terminal complement components on erythrocytes are activated, causing cell hemolysis.

Downey cell outdated term used to describe morphologic variations of the reactive lymphocyte.

Drug-induced hemolysis rupture or destruction of red blood cells that occurs when a drug attaches to the erythrocyte membrane and alters it in some way.

Drug-induced hemolytic anemia hemolytic disorder precipitated by ingestion of certain drugs. The process can be immune mediated or nonimmune mediated.

Drumstick see *Barr body*.

Dry tap result of a bone marrow procedure when bone marrow cannot be aspirated. This situation can be caused by inadequate technique or alterations in the bone marrow architecture such as extensive fibrosis or very increased cellularity.

Dura mater dense membrane covering the central nervous system; outermost layer of the meninges.

Dutcher body intranuclear membrane-bound inclusion found in plasma cells. The body stains with periodic acid-Schiff (PAS), indicating that the tissue contains glycogen or glycoprotein. It appears as finely distributed chromatin, nucleoli, or intranuclear inclusions.

Dysfibrinogenemia hereditary condition in which the fibrinogen molecule has a structural alteration.

Dyshematopoiesis abnormal formation and/or development of blood cells within the bone marrow.

Dyspepsia symptom caused by abnormalities in the process of digestion.

Dysplasia abnormal cell development.

Dyspoiesis abnormal development of blood cells frequently characterized by asynchrony in nuclear to cytoplasmic maturation and/or abnormal granule development.

Ecchymosis bruise (bluish-black discoloration of the skin) that is larger than 3 mm in diameter caused by bleeding from arterioles into subcutaneous tissues without disruption of intact skin.

Echinocyte spiculated erythrocyte with short, equally spaced projections over the entire outer surface of the cell.

Edematous marked by the swelling of body tissues from the accumulation of tissue fluid.

Effector lymphocyte antigen-stimulated white blood cell that mediates the efferent arm of the immune response.

Effector T lymphocyte T lymphocyte that has encountered antigen and undergone blast transformation; morphologically indistinguishable from original T lymphocyte.

Efficacy ability to produce the desired effect (e.g., anticoagulation).

Effusion abnormal accumulation of fluid.

Egress action of going out or exiting; describes the exit of blood cells from the blood to the tissue.

ELISA *See* Enzyme-linked immunosorbent assay.

Elliptocyte abnormally shaped erythrocyte that is an oval to elongated ellipsoid with a central area of pallor and hemoglobin at both ends. Also known as *ovalocyte, pencil cell,* and *cigar cell.*

Embolism blockage of an artery by a piece of blood clot or other foreign matter, resulting in obstruction of blood flow to the tissues.

Embolus piece of blockage or other foreign matter that circulates in the blood stream and usually becomes lodged in a small vessel obstructing blood flow.

Endomitosis round of nuclear DNA synthesis without nuclear or cytoplasmic division.

Endoplasmic reticulum (ER) cytoplasmic organelle in eukaryotic cells that consists of a network of interconnected tubes and flattened membranous sacs. If the ER has ribosomes attached, it is known as *granular* or *rough endoplasmic reticulum* (*RER*), and if ribosomes are not attached, it is known as *smooth endoplasmic reticulum* (*SER*).

Endosteum membrane that lines the bone medullary cavity that contains the bone marrow.

Endothelial cell flat cells that line the cavities of the blood and lymphatic vessels, heart, and other related body cavities.

Endothelial cell protein C receptor (EPCR) molecule on the membrane of endothelial cells of larger vessels that binds and immobilizes protein C, augmenting its activation by the thrombin: thrombomodulin complex.

End point detection place where the completion of a reaction is marked by some change such as formation of a clot or color change.

Engraftment process of infusing stem cells into the bone marrow microenvironment resulting in hematopoietic recovery.

Enzyme protein that catalyzes a specific biochemical reaction but is not itself altered in the process.

Enzyme-linked immunosorbent assay analysis used to test for proteins using immunologic principles; one use in coagulation testing is to detect antibodies against heparin–PF4 complexes.

Eosinophil mature granulocyte cell characterized by the presence of large acidophilic granules that are pink to orange pink with Romanowsky stains. The cell is 12–17 mcM (μm) in diameter, and the nucleus has 2–3 lobes. Granules contain acid phosphatase, glycuronidase cathepsins, ribonuclease, arylsulfatase, peroxidase, phospholipids, and basic proteins. Eosinophils have a concentration of $<0.45 \times 10^9$/L in the peripheral blood. The cell membrane has receptors for IgE and histamine.

Eosinophilia increase in the concentration of eosinophils in the peripheral blood ($>0.5 \times 10^9$/L) associated with parasitic infection, allergic conditions, hypersensitivity reactions, cancer, and chronic inflammatory states.

Epigenetic related to heritable changes in gene expression not caused by changes in DNA sequence.

Epistaxis hemorrhage from the nose.

Epitope structural portion of an antigen that reacts with a specific antibody. Also called *antigenic determinant.*

Epstein-Barr virus (EBV) infectious agent that attaches to the membrane surface of B lymphocytes by a specific receptor designated CD21.

Error detection identification of a laboratory's multirule quality control procedure to detect a true mistake in the testing system and reject the control run.

Erythroblast nucleated erythrocyte precursor in the bone marrow. If normal, it can be referred to as *normoblast.*

Erythroblastic island composite of erythroid cells in the bone marrow that surrounds a central macrophage. These cell groups are usually disrupted when bone marrow smears are made but can be found in erythroid hyperplasia. The least mature cells are closest to the center and the more mature cells are on the periphery. The central macrophage is thought to transfer iron to the developing cells.

Erythroblastosis fetalis hemolytic anemia in newborns caused by an antigen–antibody reaction when fetal-maternal blood group incompatibility involves the Rh factor or ABO blood groups. Antibody from the mother traverses the placenta and attaches to antigens on the fetal cells.

Erythrocyte red blood cell (RBC) that has matured to the nonnucleated stage. The cell is about 7 mcM (μm) in diameter and contains the respiratory pigment hemoglobin, which readily combines with oxygen to form oxyhemoglobin. The cell develops from the pluripotential stem cell in the bone marrow under the influence of the hematopoietic growth factor erythropoietin and is released to the peripheral blood as a reticulocyte. Its average life span is about 120 days after which it is removed by cells in the mononuclear–phagocyte system. The average concentration is about 5×10^{12}/L for males and 4.5×10^{12}/L for females.

Erythrocytosis abnormal increase in the number of circulating erythrocytes as measured by the erythrocyte count, hemoglobin, or hematocrit.

Erythron summation of the stages of erythrocytes in the marrow or peripheral blood and within vascular areas of specific organs such as the spleen.

Erythrophagocytosis phagocytosis of an erythrocyte by a histiocyte that can be seen within its cytoplasm as a pink globule or, if digested, a clear vacuole on stained bone marrow or peripheral blood smears.

Erythropoiesis formation and maturation of erythrocytes in the bone marrow. It is under the influence of the hematopoietic growth factor erythropoietin.

Erythropoietin hormone secreted by the kidney that regulates erythrocyte production by stimulating the stem cells of the bone marrow to mature into erythrocytes. Its primary effect is on the committed stem cell CFU-E.

Essential thrombocythemia myeloproliferative neoplasm primarily affecting the megakaryocytic element in the bone marrow. There is extreme thrombocytosis in the blood (usually $>1000 \times 10^9$/L). Also called *primary thrombocythemia, hemorrhagic thrombocythemia,* and *megakaryocytic leukemia.*

Euchromatin region of the chromosome that contains genetically active DNA, is light staining, and replicates early in the cell cycle's S phase. *See* Heterochromatin.

Evan's syndrome condition characterized by a warm autoimmune hemolytic anemia and concurrent severe thrombocytopenia.

Exchange transfusion simultaneous withdrawal of blood and infusion with compatible blood.

Exon protein-coding DNA sequence of a gene.

Extracellular matrix noncellular component of the hematopoietic microenvironment in the bone marrow.

Extramedullary erythropoiesis red blood cell production occurring outside the bone marrow.

Extramedullary hematopoiesis formation and development of blood cells at a site other than the bone marrow.

Extravascular occurring outside of the blood vessels.

Extrinsic pathway one of the three interacting coagulation factor sequences in the coagulation cascade initiated when tissue factor comes into contact with blood and forms a complex with FVII. The complex activates FX. The term *extrinsic* is used because the sequence of factor activation requires tissue factor, a factor outside of blood.

Extrinsic Xase complex complex of tissue factor and FVIIa that forms when a vessel is injured and serves to activate FX.

Exudate effusion formed by increased vascular permeability and/or decreased lymphatic resorption; indicates a true pathologic state in the anatomic region, usually either infection or tumor.

FAB classification *See* French-American-British classification.

Factor V Leiden (FVL) single point mutation of the FV gene (*F5*), involving replacement of Arg 506 with Gln(FV^{R506Q}) that makes the mutant FVa molecule resistant to inactivation by APC by altering an APC cleavage site and leads to thrombophilia; FV serves as a cofactor in the APC inactivation of FVIIIa. FVL is a much less effective cofactor in this inactivation of FVIIIa, which contributes to the thrombophilic state.

Factor VIII: C assay method that determines the amount of FVIII.

Factor VIII concentrate lyophilized preparation of condensed F-VIII used for replacement therapy of FVIII in patients with hemophilia A.

Factor VIII inhibitor IgG immunoglobulin with antibody specificity to FVIII that inactivates the factor. The antibodies are time and temperature dependent. It is associated with hemophilia.

Factor VIII/VWF complex plasma form of VWF associated with FVIII.

Faggot cell a neoplastic myeloid precursor that has a large collection of Auer rods and/or phi bodies.

False rejection failure of a control run that is not truly out of control whose result falls outside the control limits or violates a Westgard rule; is caused by the inherent imprecision of the test method.

Fanconi anemia (FA) autosomal recessive disorder characterized by chromosomal instability. Patients have a complex assortment of congenital anomalies in addition to a progressive bone marrow hypoplasia.

Favism sensitivity to a species of bean, *Vicia faba*; commonly found in Sicily and Sardinia in individuals who have inherited glucose-6-phosphate dehydrogenase (G6PD) deficiency. Its characteristics are fever, acute hemolytic anemia, vomiting, and diarrhea after ingestion of the bean or inhalation of the plant pollen.

Fee for service payment method for health care in which consumers choose their own health care providers and the provider determines the fees for the services. The patient or a third-party payer pay the fees.

Ferritin iron-phosphorus-protein that forms a compound when iron complexes with the protein apoferritin. It is a storage form of iron found primarily in the bone marrow, spleen, and liver. Small amounts can be found in the peripheral blood proportional to that found in the bone marrow.

Ferroportin protein that transports ferrous iron across the basolateral membrane of enterocytes. The only known cellular exporter of iron, it is involved in the membrane transport of iron from macrophages and hepatocytes. Also known as *IREG1*.

Fibrin degradation product (FDP) breakdown product of fibrin or fibrinogen that is produced when plasmin's proteolytic action cleaves this molecule. The four main products are fragments X, Y, D, and E. The presence of fibrin degradation products indicates either fibrinolysis or fibrinogenolysis.

Fibrin monomer structure resulting when thrombin cleaves the A and B fibrinopeptides from the α- and β-chains of fibrinogen.

Fibrinogen group assemblage of coagulation factors activated by thrombin and consumed during the formation of fibrin and therefore absent from serum. It includes factors I, V, VIII, and XIII; also called *consumable group*.

Fibrinolysis breakdown of fibrin.

Fibrin polymer complex of covalently bonded fibrin monomers. The bonds between glutamine and lysine residues are formed between terminal domains of γ-chains and polar appendages of α-chains of neighboring residues.

Fibronectin extracellular-matrix glycoprotein capable of binding heparin.

Fibrosis abnormal formation of fibrous tissue.

Flame cell an abnormal plasma cell with reddish-purple cytoplasm. The red tinge is caused by the presence of a glycoprotein and the purple of ribosomes.

Flow chamber specimen-handling area of a flow cytometer where cells are forced into single file and directed in front of the laser beam.

Fluorescence in situ hybridization (FISH) technique in which whole chromosomes (metaphase or interphase) are hybridized to a complementary probe that is labeled with a fluorochrome and visualized by microscopy.

Fluorochrome molecule excited by light of one wavelength and emits light of a different wavelength.

Folate large group of compounds whose parent substance is folic acid; composed of three parts: peteridine, p-amino-benzoic acid, and chain of glutamic acid residues. The function of the active form, tetrahydrofolate (THF), is to transfer one-carbon compounds from donor molecules to acceptor molecules in intermediary metabolism.

Folic acid parent substance of folates.

Forward light scatter laser light spread in a forward direction in a flow cytometer; is related to particle size (e.g., large cells produce more forward spread than smaller cells).

Free erythrocyte protoporphyrin (FEP) protoporphyrin within an erythrocyte that is not complexed with iron. Its concentration increases in iron-deficient states. In the absence of iron, erythrocyte protoporphyrin combines with zinc to form zinc protoporphyrin (ZPP).

French-American-British (FAB) classification system for hematopoietic disorders based on cell lineage as determined by the morphology and results of cytochemical stains; has been replaced by the WHO 2008 classification.

Fresh frozen plasma (FFP) colorless fluid portion of blood that is frozen at −18° C or colder within 6 hours of collection; formed by removal of all cellular components.

F-test statistical tool used to compare features of two or more sets of data.

Functional anemia decrease in hemoglobin concentration accompanied by a decrease in oxygen delivery to the tissues.

Functional hyposplenism reduced splenic function not caused by the loss of splenic tissue but by the accumulation of cells sequestered in the spleen.

Functional iron-deficiency anemia disorder caused by a lack of iron because it cannot be mobilized from storage sites to meet the need of developing erythroblasts.

γ-Carboxylation a post-translational modification of a protein that adds an extra carboxyl group (COOH) to the γ-carbon of the glutamic acid residues; requires reduced vitamin K; the prothrombin group of coagulation factors as well as protein C, S, and Z require this conversion to become active

Gammopathy abnormal condition with an increase in serum immunoglobulins.

Gating in flow cytometry, the process of isolating cells with the same light-scattering or fluorescence properties by electronically placing a gate around them.

Gene functional segment of DNA that serves as a template for RNA transcription and protein translation. Regulatory sequences control its expression so that only a small fraction of the estimated 100,000 units is ever transcribed by a given cell.

Gene cluster group of closely linked units of heredity that can be affected as a group.

Gene promoter DNA sequence that RNA polymerase binds to in order to begin transcription of a unit of heredity.

Gene rearrangement process in which segments of DNA are cut and spliced to produce new DNA ones. During normal lymphocyte development, reorganization of the immunoglobulin and the T-cell receptor DNAresults in new sequences that encode the antibody and surface antigen receptor proteins necessary for immune function.

Gene therapy treatment that places a normally functioning unit of heredity into an affected individual's appropriate target cell.

Genome total aggregate of inherited genetic material that in humans consists of 3 billion base pairs of DNA divided among 46 chromosomes including 22 pairs of autosomes numbered 1–22 and the two sex chromosomes.

Genomics study of all nucleotide sequences, including structural units of heredity, regulatory sequences, and noncoding DNA segments in an organism's chromosomes.

Genotype hereditary constitution of an individual, often referring to a particular DNA locus.

Germinal center lightly staining core of a lymphoid follicle where B-cell activation occurs.

Germline cell lineage that pass their genetic material to progeny

Glanzmann thrombasthenia rare hereditary platelet disorder characterized by a genetic mutation in one of the genes coding for the glycoproteins IIb or IIIa and resulting in the inability of platelets to aggregate.

Global testing analyzing the entire hemostatic process including coagulation, anticoagulant effects, fibrin formation and stabilization, clot retraction, and fibrinolysis on a whole blood sample by use of a specialized instrument.

Globin protein portion of the hemoglobin molecule.

Glossitis inflammation of the tongue.

Glucose-6-phosphate-dehydrogenase (G6PD) enzyme within erythrocytes that is important in carbohydrate metabolism. It dehydrogenates glucose-6-phosphate to form 6-phosphogluconate in the hexose monophosphate shunt. This reaction produces NADPH from NADP and provides reducing power to the erythrocyte, protecting the cell from oxidant injury.

Glutathione tripeptide that takes up and gives up hydrogen and prevents oxidant damage to the hemoglobin molecule. A deficiency of it is associated with hemolytic anemia.

Glycocalin portion of glycoprotein Ib of the platelet membrane external to the platelet surface; contains binding sites for von Willebrand factor and thrombin.

Glycocalyx amorphous coat of glycoproteins and mucopolysaccharides covering the surface of cells, particularly the platelets and endothelial cells.

Glycolysis anaerobic conversion of glucose to lactate and pyruvic acid resulting in the production of energy (ATP).

Glycoprotein Ib a substance on the platelet surface that contains the receptor for von Willebrand factor and is critical for initial adhesion of platelets to collagen after an injury.

Glycoprotein IIb/IIIa complex of membrane proteins on the platelet surface that is functional only after activation by agonists and then becomes a receptor for fibrinogen and von Willebrand factor. It is essential for platelet aggregation.

Glycosylated hemoglobin hemoglobin (HbA_{1c}) that has glucose irreversibly attached to the terminal amino acid of the β-chains.

Golgi apparatus cytoplasmic organelle composed of flattened sacs or cisternae arranged in stacks. It functions to concentrate and package the products of secretory cells. It does not stain with Romanowsky stains and appears as a clear area usually adjacent to the nucleus.

Gower hemoglobin embryonic intracellular erythrocyte protein that is detectable in the yolk sac for up to 8 weeks of gestation; composed of two zeta (ζ) chains and two epsilon (ϵ) chains.

Graft-versus-host disease (GVHD) tissue injury secondary to HLA-mismatch grafts resulting from immunocompetent donor T lymphocytes that recognize HLA antigens on the host cells and initiate a secondary inflammatory response.

Graft versus leukemia favorable effect seen when immunocompetent donor T cells present in the allograft destroy the recipient's leukemic cells.

Granulocytopenia decrease in neutrophils below the reference range (1.8×10^9/L).

Granulocytosis increase in neutrophils above the reference range (7.0×10^9/L), usually seen in bacterial infections, inflammation, metabolic intoxication, drug intoxication, and tissue necrosis.

Granulomatous distinctive pattern of chronic reaction in which the predominant cell type is an activated macrophage with epithelial-like (epithelioid) appearance.

Gray platelet syndrome (α-storage pool disease) rare hereditary disorder characterized by the lack of α-granules of thrombocytes.

Hairy cell neoplastic cell of hairy cell leukemia characterized by circumferential, cytoplasmic, hairlike projections.

Ham test specific laboratory test for paroxysmal nocturnal hemoglobinuria (PNH). When erythrocytes from a patient with PNH are incubated in acidified serum, the cells lyse as the result of complement activation. Also called *acid-serum lysis test.*

Haploid having an n number of chromosomes in a gamete; consisting of one of each of the autosomes and one of the sex chromosomes. For human cells, it is 23; n = 23.

Haplotype one of the two alleles at a genetic locus.

Haptoglobin serum α_2-globulin glycoprotein that transports free plasma hemoglobin to the liver.

HDFN *See* Hemolytic disease of the fetus and newborn.

Health Insurance Portability and Accountability Act (HIPAA) law that mandates health care entities to establish measures that ensure the confidentiality of patient information.

Heinz body substance in the erythrocyte composed of denatured or precipitated hemoglobin; appears as purple-staining body on supravitally stained (crystal violet) blood smears.

HELLP syndrome *See* Hemolysis, elevated liver enzymes and low platelet (HELLP) syndrome.

Helper T cell (T_H) effector lymphocyte subset that provides helper activity for B cells, macrophages, and other T cells; subsets include T_H1, T_H2, and T_H17.

Helmet cell abnormally shaped erythrocyte with one or several notches and projections on either end that look like horns. The shape is caused by trauma to the erythrocyte. Also called *keratocyte* and *horn-shaped cell.*

Hematocrit packed cell volume of erythrocytes in a given volume of blood following centrifugation expressed as a percentage of total blood volume or as a liter of erythrocytes per liter of blood (L/L). Also referred to as *packed cell volume* (*PCV*).

Hematogone precursor B lymphocyte present normally in the bone marrow.

Hematologic remission in a patient treated for a hematologic neoplasm, it is the state of absence of neoplastic cells in the peripheral blood and bone marrow and the return to normal levels of hematologic parameters.

Hematology study of formed cellular blood elements.

Hematoma localized collection of blood under the skin or in other organs caused by a break in a blood vessel wall.

Hematopoiesis production and development of blood cells normally occurring in the bone marrow under the influence of hematopoietic growth factors.

Hematopoietic microenvironment specialized, localized surrounding in hematopoietic organs that supports the development of hematopoietic cells.

Hematopoietic progenitor cell blood precursor cell developmentally located between stem cells and the morphologically recognizable blood precursor cells; includes multilineage and unilineage cell types.

Hematopoietic stem cell blood precursor cell capable of giving rise to all lineages of blood cells.

Heme nonprotein portion of hemoglobin and myoglobin that contains iron nestled in a hydrophobic pocket of a porphyrin ring (ferroprotoporphyrin); responsible for the characteristic color of hemoglobin.

Hemochromatosis clinical condition resulting from abnormal iron metabolism characterized by accumulation of iron deposits in body tissues.

Hemoconcentration increased concentration of blood components from loss of plasma from the blood.

Hemoglobin intracellular erythrocyte protein that is responsible for transporting oxygen and carbon dioxide between the lungs and body tissues.

Hemoglobin distribution width measure of the distribution of intracellular erythrocyte protein within an erythrocyte population. It is derived from the hemoglobin histogram generated by the Bayer/Technicon instruments.

Hemoglobin electrophoresis method of identifying intracellular erythrocyte proteins based on differences in their electrical charges.

Hemoglobinemia presence of hemoglobin in the plasma.

Hemoglobinopathy disease that results from an inherited abnormality of the structure or synthesis of the globin portion of the hemoglobin molecule.

Hemoglobinuria presence of free hemoglobin in the urine.

Hemojuvelin (HJV) glycosylphosphatidylnositol-anchored protein that has been shown to regulate hepcidin expression.

Hemolysis destruction of erythrocytes resulting in the release of hemoglobin. In hemolytic anemia, the premature destruction of erythrocytes.

Hemolysis, elevated liver enzymes and low platelet (HELLP) syndrome obstetric disorder characterized by hemolysis (H), elevated liver enzymes (EL), and a low platelet count (LP); its etiology and pathogenesis are not well understood. It can cause microangiopathic hemolytic anemia.

Hemolytic anemia disorder characterized by a decreased erythrocyte concentration from premature destruction of the erythrocyte.

Hemolytic disease of the fetus and newborn (HDFN) alloimmune disease characterized by fetal red blood cell destruction as a result of incompatibility between maternal and fetal blood groups.

Hemolytic transfusion reaction one of two types of interaction between foreign (nonself) erythrocyte antigens and plasma antibodies from a transfusion of blood: immediate (within 24 hours) and delayed (occurring 2 to 14 days after transfusion).

Hemolytic uremic syndrome (HUS) disorder characterized by a combination of microangiopathic hemolytic anemia, acute renal failure, and thrombocytopenia.

Hemopexin plasma glycoprotein (β-globulin) that binds the heme molecule in plasma in the absence of haptoglobin.

Hemophilia A sex-linked (X-linked) hereditary hemorrhagic disorder caused by a genetic mutation of the gene coding for coagulation FVIII.

Hemophilia B sex-linked (X-linked) hereditary hemorrhagic disorder caused by a genetic mutation of the gene coding for coagulation FIX.

Hemorrhage loss of a large amount of blood either internally or externally.

Hemorrhagic disease of the newborn severe bleeding disorder in the first week of life caused by deficiency of vitamin K.

Hemosiderin water insoluble, heterogeneous iron–protein complex found primarily in the cytoplasm of cells (normoblasts and histiocytes in the bone marrow, liver, and spleen); the major long-term storage form of iron. It is readily visible microscopically in unstained tissue specimens as irregular aggregates of golden yellow to brown granules. It can be visualized with Prussian-blue stain as blue granules that are normally distributed randomly or diffused.

Hemosiderinuria presence of iron-containing protein in the urine as a result of intravascular hemolysis and disintegration of renal tubular cells.

Hemostasis localized, controlled process that arrests bleeding after an injury.

Heparin polysaccharide that inhibits coagulation of blood by preventing thrombin from cleaving fibrinogen to form fibrin; commercially available in the form of a sodium salt for therapeutic use as an anticoagulant.

Heparin-associated thrombocytopenia (HAT) abnormal drop in the number of platelets associated with specific anticoagulant (heparin) therapy in some patients from a nonimmune-mediated direct platelet activation effect.

Heparin-induced thrombocytopenia (HIT) abnormal drop in the number of blood cells involved in forming blood clots associated with anticoagulant (heparin) therapy in some patients resulting from an immune-mediated destruction of these cells from drug (heparin)-dependent platelet-activating IgG antibodies produced against the platelet factor 4 (PF4)-heparin complex.

Hepcidin master iron-regulating protein that regulates iron recycling/balance via interaction with ferroportin; a negative regulator of intestinal iron absorption.

Hephaestin protein that facilitates export of iron across the basolateral enterocyte membrane and oxidizes Fe^{++} iron to Fe^{+++} for binding to apotransferrin.

Hereditary elliptocytosis autosomal-dominant condition characterized by the presence of increased numbers of elongated and oval erythrocytes. The abnormal shape results from a horizontal interaction defect with abnormal spectrin, deficiency, or defect in band 4.1 or deficiency of glycophorin C and abnormal band 3.

Hereditary erythroblastic multinuclearity with positive acidified serum test (HEMPAS) congenital erythrocytic multinuclearity with positive acidified serum lysis that is type II congenital dyserythropoietic anemia (CDA), which is characterized by both abnormal and ineffective erythropoiesis. Type II is distinguished by a positive acidified serum test but a negative sucrose hemolysis test.

Hereditary pyropoikilocytosis (HPP) rare but severe hemolytic anemia inherited as an autosomal recessive disorder. It is characterized by marked erythrocyte fragmentation. The defect is most likely a spectrin abnormality in the erythrocyte cytoskeleton.

Hereditary spherocytosis chronic hemolytic anemia caused by an inherited erythrocyte membrane disorder. The vertical interaction defect is most commonly the result of a combined spectrin and ankyrin deficiency. The defect causes membrane instability and progressive membrane loss. Secondary to membrane loss, cells become spherocytes and are prematurely destroyed in the spleen. The condition is usually inherited as an autosomal dominant trait.

Hereditary stomatocytosis rare hemolytic anemia inherited in an autosomal dominant fashion. The erythrocyte membrane is abnormally permeable to cations. The cell becomes overhydrated, resulting in the appearance of stomatocytes or dehydration resulting in the appearance of targeted or contracted and spiculated cells. Also called *hereditary hydrocytosis*. See *overhydrated hereditary stomatocytosis, OHS; dehydrated hereditary stomatocytosis, DHS*.

Hereditary xerocytosis congenital disorder in which the erythrocyte is abnormally permeable to sodium and potassium with an increased potassium efflux. The erythrocyte becomes dehydrated and appears as either a target or spiculated cell. The cell is rigid and becomes trapped in the spleen. Now referred to as dehydrated hereditary stomatocytosis (DHS).

Heterochromatin region of the chromosome that contains genetically inactive DNA, is dark staining, and replicates late in the S phase of the cell cycle.

Heterologous related to morphologically nonidentical chromosomes that have different gene loci.

Heterophile antibody immunoglobulin that can react against a heterologous antigen that did not stimulate the production of immunoglobulin. In infectious mononucleosis, this immunoglobulin is in response to infection with Epstein-Barr virus and react with sheep, horse, and beef erythrocytes.

Heterozygous related to different genes at a gene locus.

Hexose-monophosphate shunt (HMP) metabolic pathway that converts glucose-6-phosphate to pentose phosphate. This pathway couples oxidative metabolism with the reduction of nicotinamide adenine dinucleotide-phosphate (NADPH) and glutathione, which provides the cell with reducing power and prevents injury by oxidants.

HFE *See* human hemochromatosis protein.

Histogram graphical representation of the number of cells within a defined parameter such as size.

HIV-I *See* human immunodeficiency virus type I.

Hodgkin lymphoma malignancy that most often arises in lymph nodes and is characterized by the presence of Reed-Sternberg cells and variants with a background of varying numbers of benign lymphocytes, plasma cells, histiocytes, and eosinophils. The malignant cell's origin is still controversial.

Homologous pertaining to two morphologically identical chromosomes that have identical gene loci but can have different gene alleles because one member of the pair is of maternal origin and the other is of paternal origin.

Homozygous pertaining to identical genes at a gene locus.

Horizontal interaction side-by-side interrelationship involving the proteins of the erythrocyte membrane.

Howell-Jolly body erythrocyte inclusion composed of nuclear remnants (DNA). On Romanowsky-stained blood smears, it appears as a dark purple spherical granule usually near the cell's periphery. It is commonly associated with megaloblastic anemia and splenectomy.

Human hemochromatosis protein (HFE) transmembrane protein that associates with β2-microglobulin. It binds to the transferrin receptor (TfR) on cells. It is involved in regulating iron absorption and uptake. Mutations are associated with a decrease in hepcidin, resulting in hemochromatosis.

Human immunodeficiency virus type I virus that causes acquired immunodeficiency syndrome (AIDS).

Human platelet antigen unique alloantigens associated with the surface of the blood cells involved in clotting.

Humoral immunity protection imparted as a result of B lymphocyte activation. The B lymphocyte differentiates to a plasma cell that produces antibodies specific to the antigen that stimulated the response.

Hybridization process in which one nucleotide strand binds to another strand by forming hydrogen bonds between complementary nucleotides.

Hydrodynamic focusing technique that allows cells/particles to flow in a single column because of differences in the pressures of two columns of fluid in a flow chamber of a flow cytometer. The particles are contained in an inner column of sample fluid surrounded by a column of stream sheath fluid. The gradient between the sample and sheath fluid keeps them separate (laminar flow) and is used to control the diameter of the column of sample fluid. The central column of sample fluid is narrowed to isolate single cells that pass through a laser beam like a string of beads.

Hydrops fetalis genetically determined hemolytic disease (thalassemia) resulting in production of an abnormal hemoglobin (hemoglobin Bart's, γ_4) that is unable to carry oxygen. No alpha (α) globin α-chains are synthesized. There is gross edema of the entire body.

Hypercoagulable state condition associated with an imbalance between clot-promoting and clot-inhibiting factors. leading to an increased risk of developing thrombosis.

Hyperdiploid having $>$2n chromosomes per cell; for human cells, this would be $>$46.

Hypereosinophilic syndrome persistent blood eosinophilia $>1.5 \times 10^9$/L with tissue infiltration, absence of clonal genetic aberrations, and no apparent cause of the increase in eosinophils.

Hyperhomocysteinemia medical condition characterized by elevated level of homocysteine in the blood as a result of impaired homocysteine metabolism. It can be due to acquired or congenital causes. It is associated with premature atherosclerosis and arterial thrombosis.

Hyperplasia enlargement of an organ or tissue caused by an increase in the number of cells per unit volume of tissue. This can result from an increase in the number of cells replicating, an increase in the rate of replication, or prolonged survival of cells. The cells usually maintain normal size, shape, and function. The stimulus for the proliferation can be acute injury, chronic irritation, or prolonged, increased hormonal stimulation. In hematology, a hyperplastic bone marrow is one in which the proportion of hematopoietic cells to fat cells is increased.

Hypersplenism disorder characterized by enlargement of the spleen and pancytopenia in the presence of a hyperactive bone marrow.

Hypocellularity decreased state of hematopoietic precursors in the bone marrow.

Hypochromic pertaining to lack of color in erythrocytes with an enlarged area of pallor caused by a decrease in the cell's hemoglobin content. The mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) are decreased.

Hypodiploid having $<$2n of chromosomes per cell; for human cells, this would be $<$46.

Hypofibrinogenemia condition in which there is an abnormally low fibrinogen level in the peripheral blood. It can be caused by a mutation in the gene controlling the production of fibrinogen or an acquired condition in which fibrinogen is pathologically converted to fibrin.

Hypogammaglobulinemia condition associated with a decrease in resistance to infection as a result of decreased γ-globulins (immunoglobulins) in the blood.

Hypoplasia condition of underdeveloped tissue or organ usually caused by a decrease in the number of cells. This bone marrow type is one in which the proportion of hematopoietic cells to fat cells is decreased.

Hypoproliferative having decreased production of any cell type.

Hypoxia deficiency of oxygen to the cells.

Hypoxia-inducible factor-2 (HIF-2) transcription factor that regulates erythropoietin synthesis and a spectrum of other hypoxic responses; activated under hypoxic conditions.

Idiopathic pertaining to disorders or diseases in which the pathogenesis is unknown.

Idiopathic thrombocytopenic purpura (ITP) *See* Immune thrombocytopenia.

Immature reticulocyte fraction (IRF) index of reticulocyte maturity provided by flow cytometry that can be helpful in evaluating bone marrow erythropoietic response to anemia, monitoring anemia, and evaluating response to therapy.

Immune hemolytic anemia disorder caused by premature, immune-mediated destruction of erythrocytes. Diagnosis is confirmed by the demonstration of immunoglobulin (antibodies) and/or complement on the erythrocytes.

Immune response body's defense mechanism that includes producing antibodies to foreign antigens.

Immune thrombocytopenia (ITP) autoimmune disorder in which autoreactive antibodies bind to platelets, shortening the platelet life span. Platelets are decreased. Also referred to as *idiopathic thrombocytopenic purpura.*

Immune tolerance immune regulatory mechanisms that govern response to self-antigens.

Immunoblast T or B lymphocyte that is mitotically active as a result of stimulation by an antigen. The cell is morphologically characterized by a large nucleus with prominent nucleoli, a fine chromatin pattern, and an abundant, deeply basophilic cytoplasm.

Immunocompetent having the ability to respond to stimulation by an antigen.

Immunoglobulin protein produced by B lymphocytes and plasma cells. It reacts with antigen and consists of two pairs of polypeptide chains: two heavy and two light chains linked together by disulfide bonds. Also called *antibody*.

Immunohistochemical stain dye applied using immunologic principles and techniques to study cells and tissues. Usually a labeled antibody is used to detect antigens (markers) on a cell.

Immunophenotyping identifying antigens using detection antibodies.

Immunosuppressed repressed ability to produce antibodies to antigens.

Immunotherapy form of treatment in which different immune cells are manipulated in vivo or in vitro and later infused to alter the immune function of other cells.

Indirect antiglobulin test (IAT) laboratory examination used to detect the presence of serum antibodies against specific erythrocyte antigens.

Induction therapy initial phase of cancer treatment using chemical substances. Its function is to rapidly drop the tumor burden and induce a remission to a normal state.

Ineffective erythropoiesis premature death of red blood cells in the bone marrow, preventing release into circulation.

Infectious lymphocytosis condition found in young children. The most striking hematologic finding is a leukocytosis of $40–50 \times 10^9$/L with 60–97% small, normal-appearing lymphocytes. It is thought to be a reactive immune response to a viral infection and is no longer considered a unique disease.

Infectious mononucleosis self-limiting lymphoproliferative disease caused by infection with Epstein-Barr virus (EBV). The usually increased leukocyte count is related to an absolute lymphocytosis. Various forms of reactive lymphocytes are present. Serologic tests to detect the presence of heterophile antibodies are helpful in differentiating this disease from more serious diseases. Also known as the *kissing disease*.

Innate immune response body's first reaction to common classes of invading pathogens. It is rapid but limited. The leukocyte receptors that participate in it are always available and do not require cell activation to be expressed. Once a pathogen is recognized, effector cells can attack, engulf, and kill it. Neutrophils, monocytes, and macrophages play a major role in the innate immune system.

In situ hybridization detection of specific DNA or RNA sequences in tissue sections or cell preparations using a labeled complementary nucleic acid sequence or probe.

Integral protein protein embedded between phospholipids within a cell membrane.

Internal quality control program system designed to verify the validity of laboratory test results as part of the daily laboratory operations. Typically, it is monitored using Levy-Jennings plots and Westgard rules.

International Classification of Disease code method for indicating medical diagnosis, conditions, symptoms, and cause of death in all health care settings; accepted worldwide. One of its uses is to help evaluate medical necessity and determine reimbursement for tests and procedures,

International normalized ratio (INR) method of reporting prothrombin time results when monitoring long-term oral anticoagulant therapy. Results are independent of the reagents and methods used.

International sensitivity index (ISI) list of values provided by the manufacturer of thromboplastin reagents indicating the responsiveness of the particular lot of reagent compared to the international reference thromboplastin.

Intrinsic coagulation pathway one of the three interacting sequences of coagulation factors in the coagulation cascade. The intrinsic passage sequence is initiated by exposure of the contact coagulation factors (FXII, FXI, prekallikrein, and high-molecular-weight kininogen) with vessel subendothelial tissue. The intrinsic sequence activates FX.

Intrinsic factor glycoprotein secreted by the parietal cells of the stomach that is necessary for binding and absorption of dietary cobalamin (vitamin B_{12}).

Intrinsic pathway *See* Intrinsic coagulation pathway.

Intrinsic Xase complex network of FIXa, FVIIIa, phospholipid, and calcium that assembles on membrane surfaces.

Intron DNA base sequence that interrupts the protein coding sequence of a gene; this sequence is transcribed into RNA but is cut out of the message before it is translated into protein.

Iron-deficiency anemia microcytic, hypochromic disorder caused by a deficiency of total body iron, resulting in iron-restricted erythropoiesis. This is the last stage of the disorder when all laboratory tests for iron status become abnormal.

Iron-responsive element (IRE) stem-loop-stem structure in either the 5′ or 3′ noncoding regions of mRNA recognized and bound by iron-binding proteins (IRE-BP or iron-regulatory protein [IRP]). The IRP binding affinity for the IRE is determined by the amount of cellular iron; the IRP binds to the IRE region when iron is scarce and dissociates when it is plentiful. When bound, the IRP modulates the translation of the mRNA. The translation of the proteins involved in iron metabolism including ferritin, ferroportin, ALAS2, transferrin, and DMT1 is regulated by this mechanism.

Iron-responsive element-binding protein (IRE-BP) protein that binds to a stem-loop structure of some mRNAs which is known as the *iron-responsive element (IRE)*. The IRE-BP binding affinity for the IRE is determined by the amount of cellular iron and is involved in the regulation of translation of proteins involved in iron metabolism.

Irreversibly sickled cell (ISC) rigid cell that has been exposed to repeated sickling events and cannot revert to a normal discoid shape. It is ovoid or boat shaped and has a high MCHC and low MCV.

Ischemia deficiency of blood supply to a tissue caused by constriction of the vessel or blockage of the blood flow through it.

Isoelectric focusing technique of moving charged particles through a support medium with a continuous pH gradient. Individual proteins move until they reach the pH that is equal to their isoelectric point.

Isopropanol precipitation technique that identifies the presence of unstable hemoglobins because of their insolubility in isopropanol as compared to normal hemoglobins.

Isovolumetric sphering method employed by the Bayer/Technicon instruments in which a specific buffered diluent is used to sphere and fix the blood cells without altering their volume.

***JAK2* gene** heredity unit that codes for a tyrosine kinase closely associated with cytokine receptors. A gain in function mutation that gives the cell a proliferative advantage is common in the myeloproliferative disorders, especially polycythemia vera.

JAK/STAT signaling system used by hematopoietic cells to relay transmissions from the cell membrane receptors to the nucleus to activate or inhibit gene expression.

Janus kinase 2 gene *See JAK2* gene.

Jaundice condition characterized by yellowing of skin, mucous membranes, and the whites of the eye caused by accumulation of bilirubin.

Juvenile myelomonocytic leukemia clonal hematopoietic neoplasm of childhood characterized by proliferation of the granulocytic and monocytic lineages. There is a peripheral blood monocytosis ($>1 \times 10^9$/L) with <20% blasts and promonocytes. Classified as a MDS/MPN disorder by the WHO 2008 classification.

Juxtacrine contact-dependent signaling used to describe a type of paracrine cell action in which a cell produces a cytokine but does not secrete it. The cytokine remains bound to the cell membrane and requires another cell to have direct cell-to-cell contact in order to achieve the desired effect.

Karyolysis destruction of the nucleus.

Karyorrhexis disintegration of the nucleus resulting in the irregular distribution of chromatin fragments within the cytoplasm.

Karyotype systematic display of a cell's chromosomes that determine the number of chromosomes present and their morphology.

Kernicterus toxic buildup of bilirubin in brain tissue; associated with hyperbilirubinemia.

Keratocyte abnormally shaped erythrocyte with one or several notches and projections on either end that look like horns. The shape is caused by trauma to the erythrocyte. Also called *helmet cells* and *horn-shaped cells.*

Killer cell cytolytic lymphocyte identified by monoclonal antibodies. It is involved in several activities such as resistance to viral infections, regulation of hematopoiesis, and process against tumor cells.

Knizocyte abnormally shaped erythrocyte that appears on stained smears as a cell with a dark, stick-shaped portion of hemoglobin in the center and a pale area on either end. The cell has more than two concavities.

L&H/popcorn cell *See* Popcorn cell.

Lacunar cell neoplastic variant found in NS Hodgkin lymphoma characterized by abundant pale-staining cytoplasm, cytoplasmic clearing, and delicate, multilobated nuclei.

Lamellar body densely packed layer of phospholipid secreted by type II alveolar cells; is the storage package for surfactant in amniotic fluid. The number of lamellar bodies present in the amniotic fluid is proportional to the amount of surfactant available

Large granular lymphocyte null cell with a low nuclear-to-cytoplasmic ratio, pale blue cytoplasm, and azurophilic granules. It does not adhere to surfaces or phagocytose.

Latex immunoassay (LIA) test immunoturbidimetric analysis using microlatex particles coated with specific antibodies. In the presence of the antigen to be tested, the particles agglutinate, producing an adsorption of light proportional to the antigen level present in the sample.

Laurell "rocket" technique (electroimmunoassay, EIA) process of electrophoresing antibody-containing agarose plates with an antigen (in PPP) until a "rocket" of precipitated antigen–antibody forms (measured following the staining of the gel). The height of the rocket is proportional to the quantity of antigen present in the PPP.

LDL receptor-like protein (LRP receptor) receiver on hepatocytes that removes plasmin/antiplasmin, plasminogen activator/plasminogen activator inhibitor, and thrombin/antithrombin complexes from the circulation.

Lecithin cholesterol acyltransferase (LCAT) deficiency rare autosomal disorder that affects the metabolism of high-density lipoproteins; characterized by the deficiency of an enzyme that catalyzes the formation of cholesterol esters from cholesterol. Its onset is usually during young adulthood.

Leptocyte abnormally shaped erythrocyte that is thin and flat with hemoglobin at the periphery. It is usually cup shaped.

Leukemia progressive, malignant disease of the hematopoietic system characterized by unregulated, clonal proliferation of hematopoietic stem cells; generally classified as chronic or acute and lymphoid or myelogenous. The malignant cells eventually replace normal cells.

Leukemia of ambiguous lineage condition in which neoplastic cells cannot be defined by one lineage. Either no lineage-specific antigens can be identified or the blasts express antigens of more than one lineage.

Leukemic hiatus gap in the normal maturation pyramid of cells with many blasts and some mature forms but very few intermediate maturational stages. Eventually, the immature neoplastic cells fill the bone marrow and spill over into the peripheral blood, producing leukocytosis (e.g., acute leukemia).

Leukemic stem cell rare cell that has infinite proliferative potential and drives the formation and growth of tumors.

Leukemogenesis process of developing a leukemic disease.

Leukemoid reaction transient, responsive condition resulting from certain types of infections or tumors characterized by an increase in the total leukocyte count to more than 25×10^9/L and a shift to the left in leukocytes (usually granulocytes).

Leukocyte white blood cell (WBC) of which there are five types: neutrophil, eosinophil, basophil, lymphocyte, and monocyte. The function of these cells is to defend against infection and tissue damage. The normal reference range for total leukocytes in peripheral blood is $4.5–11.0 \times 10^9$/L.

Leukocyte alkaline phosphatase (LAP) test that identifies the amount of enzyme present within the specific (secondary) granules of granulocytes (from the myelocyte stage onward). It is useful in distinguishing leukemoid reaction/reactive neutrophilia (high LAP) from chronic myelogenous leukemia (low LAP).

Leukocytosis increase in WBCs in the peripheral blood; WBC count $>11.0 \times 10^9$/L.

Leukoerythroblastic reaction response characterized by the presence of nucleated erythrocytes and a shift to the left in neutrophils in the peripheral blood; often associated with myelophthisis.

Leukopenia decrease in leukocytes $<4.5 \times 10^9$/L.

Leukopoiesis formation and development of white blood cells.

Linearity range of concentration over which a test method can be used without modifying (i.e., diluting) the sample.

Linearity check material commercially available matter with known concentrations of analytes that do not contain interfering substances or conditions. It is used to determine the linearity of an instrument or a method.

Linear regression analysis statistical tool used to determine a single line through a data set that describes the relationship between two methods, X and Y. General equation is $Y = a + bx$, where a denotes the y-intercept; b is the slope; and Y is the predicted mean value of Y for a given x value.

Linkage analysis process of following the inheritance pattern of a particular gene in a family based on its tendency to be inherited with another locus on the same chromosome.

Locus specific position on the chromosome.

Low-molecular-weight heparin (LMWH) heparin molecule of M.W. 2,000–12,000 daltons; used as thrombolytic therapy in acute thrombotic events.

LRP receptor *See* LDL receptor-like protein.

Lupus anticoagulant *See* Lupuslike anticoagulant.

Lupuslike anticoagulant circulating substance that occurs spontaneously in patients with a variety of conditions (originally found in those with lupus erythematosus) and directed against phospholipid components of the reagents used in laboratory tests for clotting factors. *See* Antiphospholipid antibody.

Lymphadenopathy abnormal enlargement of lymph nodes.

Lymphoblast lymphocytic precursor cell found in the bone marrow. The cell is 10–20 mcM (μm) in diameter and has a high nuclear:cytoplasmic ratio. The nucleus has a fine (lacy) chromatin pattern with one or two nucleoli. The cytoplasm is agranular and scant and stains deep blue with Romanowsky stain. The cell contains terminal deoxynucleotidyltransferase (TdT) but no peroxidase, lipid, or esterase.

Lymphocyte mature leukocyte with variable size, depending on the state of cellular activity and amount of cytoplasm. The nucleus is usually round with condensed chromatin and stains deep, dark purple with Romanowsky stains. The cytoplasm stains a light blue. Nucleoli are usually not visible. A few azurophilic granules can be present. These cells interact in a series of events that allow the body to attack and eliminate foreign antigen. They have a peripheral blood concentration in adults from 1.0 to 4.8×10^9/L 4.8×10^9/L (20–40% of leukocytes). The concentration in children less than 10 years old is higher.

Lymphocytic leukemoid reaction response characterized by an increased lymphocyte count with the presence of reactive or immature appearing lymphocytes. It is associated with whooping cough, chicken pox, infectious mononucleosis, infectious lymphocytosis, and tuberculosis.

Lymphocytopenia condition that has $<1.0 \times 10^9$/L of a type of white blood cell known as a lymphocyte. Also called *lymphopenia*.

Lymphocytosis increase in peripheral blood concentration of a type of white blood cell known as a lymphocyte ($>4.8 \times 10^9$/L in adults or $>9 \times 10^9$/L in children).

Lymphoepithelial lesion region damaged by injury or disease such as infiltration of epithelium by groups of lymphocytes. Infiltration of mucosal epithelium by neoplastic lymphocytes is characteristic of MALT lymphoma.

Lymphoid follicle spherical mass of B cells within lymphatic tissue.

Lymphokine substance released by sensitized lymphocytes and responsible for activation of macrophages and other lymphocytes.

Lymphokine-activated killer (LAK) cells subpopulation of peripheral blood mononuclear cells (natural killer cells) that have been harvested from a patient, expanded and activated with IL-2 ex vivo, and re-infused into the patient to induce an enhanced antitumor cytolytic response.

Lymphoma malignant proliferation of a type of white blood cell known as a lymphocyte. Most cases arise in lymph nodes, but it can begin at many extranodal sites; classified as to B or T cell and low, intermediate, or high grade.

Lymphoma classification process of dividing (grading) the disease into groups, each with a similar clinical course and response to treatment. Current schemes use a combination of morphologic appearance, phenotype, and genotype.

Lyonization process in which all but one X chromosome in a cell are randomly inactivated.

Lypholized freeze-dried serum or plasma sample that can be reconstituted with a diluent, typically distilled or deionized water.

Lysosomal granule small particle containing lysosomal enzymes.

Lysosomal storage disorder disruption of membrane-bound sac characterized by defects in various enzymes (including glucosidases, lipases, proteases, and nucleases) that are involved in

degradative processes leading to the accumulation of either nondegraded substrates or catabolic products that are unable to be transported out of the lysosome.

Lysosome membrane-bound sac in the cytoplasm that contains various hydrolytic enzymes.

Macrocyte abnormally large erythrocyte (>100 fL).

Macro-ovalocyte abnormally large erythrocyte with an oval shape characteristically seen in megaloblastic anemia.

Macrophage large—10–20 mcM (μm)—tissue cell derived from monocytes; it secretes a variety of products that influence the function of other cells and plays a major role in both nonspecific and specific immune responses.

Maintenance chemotherapy third and final phase of cancer treatment that uses chemical agents to prevent the repair and/or return of the malignant clone, thus allowing the normal immune system to clear away all remaining disease.

Malignant neoplastic with potential to metastasize.

Malignant neoplasm clone of identical, anaplastic (dedifferentiated), proliferating cells; can metastasize.

Marginating pool population of neutrophils attached to or marginated along the vessel walls and not actively circulating. This is about one-half of the total pool of neutrophils in the vessels.

Mastocytosis group of disorders characterized by abnormal proliferation of mast cells in one or more organs.

Material safety data sheet (MSDS) document that provides safety information for clinical laboratory professionals who use hazardous materials; includes pertinent safety information regarding the proper storage and disposal of a chemical, precautions that should be taken in handling it, potential health hazards associated with exposure to it, and whether it is a fire or explosive hazard.

Mastocytosis disorder caused by heterogeneous group of mast cell diseases characterized by the abnormal proliferation of mast cells in one or more organ systems. Two major groups of mast cell disorders are cutaneous and systemic. It is suggested that mast cell disorders be classified as myeloproliferative disorders.

Maturation process of attaining complete development of the cell.

Mature neoplasm usually a chronic condition in which the peripheral blood contains an increased total WBC and mature forms of cells.

Maturing cell committed cell that assumes the morphologic characteristics of its lineage. It makes up the majority of hematopoietic precursor cells.

May-Heggelin anomaly (MHA) inherited disorder in which platelet production is decreased; characterized by a moderate macrothrombocytopenia and Döhle-like inclusions in leukocytes; one of a group of disorders associated with abnormalities of the *MYH9* gene (nonmuscle myosin gene).

Mean cell hemoglobin (MCH) indicator of the average weight of the intracellular erythrocyte protein, hemoglobin, in individual erythrocytes reported in picograms. The reference interval for MCH is 28–34 pg. This parameter is calculated from the hemoglobin and erythrocyte count: $\text{MCH (pg)} = \dfrac{\text{Hb (g/dL)} \times 10}{\text{RBC count } (\times 10^{12}/\text{L})}$

Mean cell hemoglobin concentration (MCHC) measure of the average mass of the protein hemoglobin within erythrocytes in grams per deciliter to the volume of erythrocytes in which it is contained. The reference interval is 32–36 g/dL. The MCHC is useful when evaluating erythrocyte hemoglobin content on a stained smear. This parameter correlates with the extent of chromasia the stained cells exhibit; calculated from the hemoglobin and hematocrit:

$$\text{MCHC (g/dL)} = \frac{\text{hemoglobin (g/dL)}}{\text{hematocrit (L/L)}}$$

Mean cell volume (MCV) average volume of individual erythrocytes reported in femtoliters. The reference interval is 80–100 fL. This parameter is useful in evaluating erythrocyte morphology on a stained blood smear. The MCV usually correlates with the diameter of the erythrocytes observed microscopically and can be calculated from the hematocrit and erythrocyte count:

$$\text{MCV (fL)} = \frac{\text{hematocrit (L/L)}(\times 10^{12}/\text{L})}{\text{erythrocyte count} \times 1000}$$

Mean platelet volume average cell volume of a platelet population; analogous to the MCV of erythrocytes.

Medical decision level concentration of an analyte indicating that medical intervention is required for proper patient care.

Medullary hematopoiesis blood cell production and development in the bone marrow.

Megakaryoblast progenitor cell committed to the megakaryocyte lineage; develops into the megakaryocyte.

Megakaryocyte large cell found within the bone marrow characterized by the presence of large or multiple nuclei and abundant cytoplasm. It produces the blood platelets.

Megaloblastic asynchronous maturation of any nucleated cell type characterized by delayed nuclear development in comparison to the cytoplasmic development. The abnormal cells are large and characteristically found in pernicious anemia and other megaloblastic anemias.

Membrane inhibitor of reactive lysis (MIRL) a regulatory protein (CD59) on normal cell membranes responsible for preventing amplification of complement activation; prevents interaction between C8 and C9. Deficiency in PNH is due to the lack of a glycolipid anchor (glycosyl-phosphatidyl inositol) that attaches it to the cell membrane.

Memory cell Long-lived T or B lymphocyte produced by antigen stimulation of naïve lymphocytes that produce a rapid and enhanced immune response to the initial and later exposure to the stimulating antigen.

Mesenchymal stem cell (MSC) multipotent bone marrow stromal cells that can differentiate into bone, cartilage, and fat cells (adipocytes).

Metacentric having a chromosome that has the centromere near center so that the short arm and long arms are equal in length.

Meninges (singular: meninx) three membranes covering the brain and spinal cord.

Metamyelocyte granulocytic precursor cell normally found in the bone marrow. The cell is 10–15 mcM (μm) in diameter. The cytoplasm stains pinkish and there is a predominance of specific granules.

The nucleus is indented with a kidney bean shape, and the nuclear chromatin is condensed and stains dark purple.

Methemoglobin a form of hemoglobin with iron that has been oxidized to the ferric state; (Fe^{+++}) and is incapable of combining with oxygen.

Methemoglobinemia condition with the intracellular erythrocyte protein iron in the ferric state; this affects the oxygen affinity of the molecule, which cannot combine with oxygen.

Methemoglobin reductase pathway metabolic reaction that uses methemoglobin reductase and NADH to maintain heme iron in the reduced state (Fe^{++}).

Microangiopathic hemolytic anemia (MAHA) process in which the erythrocyte is damaged by prosthetic devices or lesions of the small blood vessels.

Microcyte abnormally small erythrocyte. The MCV is typically decreased.

Microenvironment unique surrounding in the bone marrow where orderly proliferation and differentiation of precursor cells take place.

Micromegakaryocyte small, abnormal cell sometimes found in the peripheral blood in MDS and the myeloproliferative syndromes.

Microparticle (MP) membrane-bound vesicle produced by blebbing of the parent cell's membrane released in response to apoptosis or cell activation.

Microtubule cylindric structure—20–27 mcM (μm) in diameter—composed of protein subunits. It is a part of the cytoskeleton helping some cells maintain shape. It increases during mitosis and forms the mitotic spindle fibers and assists in transporting substances in different directions. In the platelet, a band of tubules located on the circumference is thought to be essential for maintaining the disc shape in the resting state.

Minimal residual disease *See* Minimum residual disease.

Minimum residual disease condition with a combination of negative "traditional" tests (peripheral blood and bone marrow blast count and cytogenetics) and positive molecular tests for the presence of malignant cells.

Mitotic pool population of cells within the bone marrow that is capable of DNA synthesis. Also called *proliferating pool.*

Mixed lineage acute leukemia critical blood cell malignancy that has both myeloid and lymphoid populations present or blasts that possess myeloid and lymphoid markers on the same cell.

Mixed phenotype acute leukemia *See* Mixed lineage acute leukemia.

Molecular remission absence of detectable genetic abnormalities using PCR or related molecular technologies in patients who had identifiable abnormalities before therapy. This is the most sensitive test for detecting minimal residual disease.

Monoblast precursor cell found in bone marrow. It is about 14–18 mcM (μm) in diameter with abundant agranular, blue-gray cytoplasm. The nucleus can be folded or indented. The chromatin is finely dispersed, and several nucleoli are visible. The cell has nonspecific esterase activity that is inhibited by sodium fluoride. This cell will develop into monocytes.

Monoclonal gammopathy alteration in immunoglobulin production characterized by an increase in one specific class of immunoglobulin.

Monoclonal gammopathy of undetermined significance (MGUS) condition characterized by a low level of serum monoclonal protein without evidence of an overt neoplasm. Its diagnosis requires exclusion of other plasma cell and lymphoid malignancies.

Monocyte mature leukocyte found in bone marrow or peripheral blood. Its morphology depends on its activity. The cell ranges in size from 12–30 mcM (μm) with an average of 18 mcM. The blue-gray cytoplasm is evenly dispersed with fine dustlike granules. Of the two types of granules, one contains peroxidase, acid phosphatase, and arylsulfatase. Less is known about the content of the other granule. The nuclear chromatin is loose and linear, forming a lacy pattern. The nucleus is often irregular in shape.

Monocyte–macrophage system *See* Mononuclear phagocyte (MNP) system.

Monocytopenia disorder characterized by a decrease in the concentration of a class of circulating white blood cells known monocytes ($<0.1 \times 10^9$/L).

Monocytosis increase in the concentration of circulating white blood cells known as monocytes ($>0.8 \times 10^9$/L).

Mononuclear phagocyte (MNP) system collection of white blood cells known as monocytes and macrophages found both intravascularly and extravascularly. It plays a major role in initiating and regulating the immune response.

Monosomy condition having one daughter cell with a missing chromosome (one copy instead of two).

Morulae basophilic, irregularly shaped granular, cytoplasmic inclusions found in leukocytes in an infectious disease called *ehrlichiosis.*

Mosaic pattern that occurs in the embryo shortly after fertilization, resulting in congenital aberrations in some cells and some normal cells.

Mott cell pathologic plasma cell whose cytoplasm is filled with colorless globules that most often contain immunoglobulin (Russell bodies) and forms as a result of accumulation of material in the RER, SER, or Golgi complex caused by an obstruction of secretion. The cell is associated with chronic plasmocyte hyperplasia, parasitic infection, and malignant tumors. Also called *grape cell.*

Multimer analysis testing that determines the structure of VWF multimers.

Multiple myeloma plasma cell malignancy characterized by increased plasma proteins.

Mutation any change in the nucleotide sequence of DNA. When large sequences of nucleotides are missing, the alteration is referred to as a *deletion.*

Myeloblast first microscopically identifiable granulocyte precursor normally found in the bone marrow. The cell is large (15–20 mcM [μm]) with a high nuclear-cytoplasmic ratio. The nucleus has a fine chromatin pattern with a nucleoli. There is moderate amount of blue, agranular cytoplasm.

Myelocyte granulocytic precursor cell normally found in the bone marrow that is 12–18 mcM (μm) in diameter with a pinkish granular cytoplasm. Both primary and secondary granules are present.

Myelodysplastic syndrome (MDS) type of blood cell neoplasm characterized by a group of primary neoplastic pluripotential stem cell disorders with one or more cytopenias in the peripheral blood and prominent maturation abnormalities (dysplasia) in the bone marrow.

Myelodysplastic/myeloproliferative neoplasm category of disorders that is included in the WHO classification but not in the FAB classification system. It includes clonal hematopoietic disorders that has some clinical, laboratory, or morphologic findings of both a myelodysplastic syndrome (MDS) and a chronic myeloproliferative neoplasm (MPN).

Myeloid associated antigen CD marker that when present is unique to and useful in differentiating AML from other types of acute leukemia.

Myeloid-to-erythroid ratio (M:E ratio) proportion of granulocytes and their precursors to nucleated erythroid precursors derived from performing a differential count on bone marrow nucleated hematopoietic cells. Monocytes and lymphocytes are not included. The normal ratio is usually between 1.5:1 and 3.5:1, reflecting a predominance of myeloid elements.

Myeloid/NK cell acute leukemia acute disorder in which the neoplastic cells coexpress myeloid antigens (CD33, CD13, and/or CD15) and NK cell–associated antigens (CD56, CD16, CD11b) although they lack HLA-DR and T lymphocyte–associated antigens CD3 and CD8.

Myeloperoxidase enzyme present in the primary granules of myeloid cells including neutrophils, eosinophils, and monocytes.

Myelophthisis displacement of normal hematopoietic tissue in bone marrow by fibrosis, leukemia, or metastatic cancer cells.

Myeloproliferative neoplasm (MPN) group of neoplastic clonal disorders characterized by excess proliferation of one or more cell types in the bone marrow. Formerly referred to as *myeloproliferative disorders (MPD).*

Natural killer cell type of lymphoid cell that has the capacity for spontaneous, non-MHC-restricted cytotoxicity for various target cells. It possesses CD16 (the FcγIII receptor for IgG) and CD56. NK cells constitute about 15% of the circulating lymphocytes in the peripheral blood.

Natural killer T (NKT) cell A small subpopulation of lymphocytes that express both T lymphocyte receptors (TCR) and some surface molecules characteristic of natural killer cells.

Necrosis pathologic cell death resulting from irreversible damage; "cell murder."

Neonatal alloimmune thrombocytopenia (NAIT) deficiency of platelets in the blood caused by immune destruction of these cells that occurs in newborns; results from the transfer of maternal alloantibodies.

Neoplasm abnormal formation of new tissue (such as a tumor) that serves no useful purpose; can be benign or malignant.

Nephelometry technique used to measure the concentration of particles in a solution by analysis of the light scattered by the particles.

Neutropenia decrease in neutrophils ($<1.8 \times 10^9$/L).

Neutrophil mature white blood cell with a segmented nucleus and granular cytoplasm. This cell constitutes the majority of circulating leukocytes. The absolute number varies between 1.8 and 7.0×10^9/L. Also called *granulocyte* or *seg.*

Neutrophilia increase in white blood cells $>7.0 \times 10^9$/L. It can be seen in bacterial infections, inflammation, metabolic intoxication, drug intoxication, and tissue necrosis.

Nondisjunction error in segregation that occurs in mitosis or meiosis so that sister chromatids do not disjoin. A spindle fiber malfunction results in one daughter cell with an extra chromosome (trisomy) and one daughter cell with a missing chromosome (monosomy).

Nonspecific esterase α-naphthyl esterase enzyme present in monocytic cells, megakaryocytes, hairy cells, plasma cells, T lymphocytes, and macrophages. Can be identified by stains using either α-naphthyl acetate or α-naphthyl butyrate as a substrate at alkaline pH.

Nonspecific granule large, blue-black particle found in promyelocytes. It has a phospholipid membrane and stains positive for peroxidase.

Nonthrombogenic pertaining to the inhibition of blood clot formation; prevent the inappropriate formation of a blood clot.

Nonthrombocytopenic purpura condition in which platelets are normal in number but purple discoloration is present; is considered to be caused by damage to the blood vessels.

Normal pooled plasma platelet-poor, colorless fluid part of blood collected from at least 20 individuals for coagulation testing. PT and APTT should give results within the laboratory's reference interval. The fluid is pooled and used in mixing studies to differentiate a circulating inhibitor from a factor deficiency.

Normoblast nucleated erythrocyte precursor in the bone marrow. Also known as *erythroblast.*

Normocytic characterized by normal size blood cells; describes erythrocytes that have a mean corpuscular volume (MCV) within the reference range (80–100 fL).

Nomogram chart that displays the relationship between numerical variables; three parallel scales are used for different variables; when a straight line connects two values, the third can be read from the point intersected by the line; used in determining heparin dosing.

Nuclear-cytoplasmic asynchrony used to describe blood cell maturation in which the cellular nucleus matures more slowly than the cytoplasm, suggesting a disturbance in coordination. As a result, the nucleus takes the appearance of one associated with a younger cell than its cytoplasmic development indicates. This is a characteristic of megaloblastic anemias.

Nuclear-to-cytoplasmic ratio (N:C ratio) proportion of the volume of the cell nucleus to the volume of the cell's cytoplasm; usually estimated as the proportion of the diameter of the nucleus to the diameter of the cytoplasm. In immature hematopoietic cells, the N:C ratio is usually higher than in more mature cells. As the cell matures, the nucleus condenses and the cytoplasm expands.

Nucleolus (pl: nucleoli) spherical body within the nucleus in which ribosomes are produced. It is not visible in cells that are not synthesizing proteins or that are not in mitosis or meiosis. It stains a lighter blue than the nucleus with Romanowsky stains.

Nucleotide basic building block of DNA composed of nitrogen base (A = adenine, T = thymine, G = guanine, or C = cytosine) attached to a sugar (deoxyribose) and a phosphate molecule.

Nucleus (pl: nuclei) characteristic structure in the eukaryotic cell that contains chromosomes and nucleoli; stains deep bluish-purple with Romanowsky stain. It is separated from the cytoplasm by a nuclear envelope. In young, immature hematopoietic cells, its material is open and dispersed in a lacy pattern. As the cell becomes mature, the structural material condenses and appears structureless.

Null cell *See* Large granular lymphocytes.

Oncogene mutated gene that leads to the transformation of a normal cell to a cancer cell. Most are altered forms of normal hereditary units that function to regulate cell growth and differentiation. Its normal counterpart is known as a *proto-oncogene.*

Open canalicular system (OCS) membrane system in the platelet forming twisted channels that lead from the platelet surface to the interior of the platelet; is a remnant of the demarcation membrane system of the megakaryocyte. Also called *surface connected canalicular system* (*SCCS*).

Opportunistic organism cell that is usually part of the normal flora but can cause disease if there is a significant change in host resistance or within the organism itself.

Opsonin antibody or complement that coats microorganisms or other particulate matter found within the blood stream so that the foreign material can be more readily recognized and phagocytized by leukocytes.

Optimal counting area region of the blood smear in which erythrocytes are just touching but not overlapping; used for morphologic evaluation and identification of cells.

Oral anticoagulant type of drug taken by mouth (e.g., Coumadin, warfarin) that prevents coagulation by inhibiting the activity of vitamin K, which is required for the synthesis of functional prothrombin group coagulation factors.

Oral anticoagulant therapy drug treatment taken by mouth to prevent coagulation.

Orthochromatic normoblast nucleated precursor of the erythrocyte that develops from the polychromatophilic normoblast normally found in the bone marrow. It is the last nucleated stage of erythrocyte development.

Osmotic fragility laboratory procedure to evaluate the ability of erythrocytes to withstand different salt concentrations; depends on the erythrocyte's membrane, volume, surface area, and functional state.

Osteoblast cell involved in formation of calcified bone.

Osteoclast cell involved in resorption and remodeling of calcified bone.

Outlier data point that falls outside the expected range for all data; is not considered to be part of the population that was sampled.

Ovalocyte erythrocyte that is oval in shape and can be seen in patients with megaloblastic and other anemias.

Overhydrated hereditary stomatocytosis (OHS) form of hereditary deformation of red blood cells in which the red cell membrane is abnormally permeable to both Na^+ and K^+. The net gain of Na^+ ions is higher than the net loss of K^+ ions because the capacity of the cation pump (fueled by ATP derived from glycolysis) to maintain normal intracellular osmolality is exceeded. As the pump fails, the intracellular concentration of cations increases, water enters the cell, and the overhydrated cells appear to have a mouthlike area of pallo and are called stomatocytes . Also known as *hereditary hydrocytosis.*

Oxygen affinity ability of hemoglobin to bind and release oxygen. It is decreased by an increase in CO_2, acid, and heat and increased by a rise in pO_2.

Oxyhemoglobin compound formed when the erythrocyte protein that transports respiratory gases combines with oxygen.

P_{50} value partial pressure of oxygen at which 50% of hemoglobin is saturated with oxygen.

***p53* gene** hereditary unit that normally functions as an antioncogene by preventing proliferation of DNA-damaged cells and unwanted DNA amplification and promoting apoptosis of these damaged cells. When mutated, it can lose its tumor-suppressive effect.

Paired *t*-test statistical tool used to compare the difference between two paired data sets. Use of pairs determines whether a statistically significant difference exists between the two data sets.

Pancytopenia marked decrease of all blood cells in the peripheral blood.

Panhypercellular increase in all blood cells in the peripheral blood.

Panic value test result that is above or below the critical limit and could pose a life-threatening situation that should be repeated to confirm the results. If confirmed, the results should be immediately communicated to the physician and properly documented.

Panmyelosis panhypercellularity in the bone marrow.

Pappenheimer body iron-containing particle in mature erythrocytes. On Romanowsky stain, it is visible near the periphery of the cell and often occurs in clusters.

Paracrine type of cell signaling when signals produced by one cell act on an adjacent cell, typically over short distances.

Paroxysmal cold hemoglobinuria (PCH) autoimmune hemolytic anemia characterized by lysis of erythrocytes and hematuria upon exposure to cold.

Paroxysmal nocturnal hemoglobinuria (PNH) stem cell disease in which the erythrocyte membrane is abnormal, making the cell more susceptible to hemolysis by complement. There is a lack of decay-accelerating factor (DAF) and MIRL on the membrane that are normally responsible for preventing amplification of complement activation. DAF and MIRL deficiency results from the lack of glycosyl phosphatidyl inositol (GPI), a membrane glycolipid that serves to attach (anchor) proteins to the cell membrane. Intravascular hemolysis is intermittent.

Passenger lymphocyte syndrome condition that causes immune hemolytic state following a solid organ, bone marrow, or stem cell transplant. The donor B lymphocytes transplanted with the organ or the bone marrow produce antibodies against recipient's blood group antigens. Hemolysis is primarily caused by ABO incompatibility between donor and recipient (Group O donor and Group A or B recipient). Although ABO is the most frequent antigen system involved, Rh, Kell, Kidd, or other blood group systems can be involved.

Pathogen-associated molecular pattern (PAMP) structure shared by many different pathogens or common alterations that the pathogen makes to the body's cells.

Pattern recognition receptor leukocyte surface responder for PAMP.

Pelger-Huët anomaly inherited benign condition characterized by the presence of functionally normal neutrophils with a bilobed or round nucleus. Cells with the bilobed appearance are called *pince-nez cells*.

Percent saturation portion of transferrin that is complexed with iron.

Pericardial cavity body space that contains the heart.

Pericardium membrane that lines the pericardial cavity.

Periodic acid-Schiff (PAS) stain used to identify and differentiate blood cells. The stainable component is primarily glycogen. Mature granulocytes, platelets, megakaryocytes, and monocytes are stained with PAS.

Peripheral membrane protein compound of amino acids attached to the cell membrane by ionic or hydrogen bonds but is outside the lipid framework of the membrane.

Peritoneal cavity space between the inside abdominal wall and outside of the stomach, small and large intestines, liver, superior aspect of the bladder, and uterus.

Peritoneum lining of the peritoneal cavity.

Pernicious anemia megaloblastic anemia resulting from a lack of intrinsic factor needed to absorb cobalamin (vitamin B_{12}) from the gut.

Persistent polyclonal B-cell lymphocytosis rare disorder found primarily in the female adult smoker. There is a polyclonal expression of lymphocytes but often there is genetic evidence of a malignant process. Hematologic findings are normal except for lymphocytosis and the presence of binucleated lymphocytes. There is a polyclonal increase in serum IgM but low IgG and IgA levels. Bone marrow examination reveals lymphocytic infiltrates.

Petechiae small, pinhead-size purple spots caused by blood escaping from capillaries into intact skin. These are associated with platelet and vascular disorders.

Phagocytosis process of cells engulfing and destroying a foreign particle through active cell membrane invagination.

Phagolysosome digestive vacuole (secondary lysosome) formed by the fusion of lysosomes and a phagosome. The hydrolytic enzymes of the lysosome digest the phagocytosed material.

Phagosome formation of an isolated vacuole within the process of opsonization.

Pharmacokinetics branch of pharmacology that performs a quantitative study of a drug's disposition in the body over time.

Phase microscopy type of light microscope used to count platelets; places an annular diaphragm below or in the substage condenser and a phase-shifting element in the rear focal plane of the objective. This causes alterations in the phases of light rays and increases the contrast between the cell and its surroundings.

Phenotype physical manifestation of an individual's genotype, often referring to a particular genetic locus.

Phi body small version of the Auer rod.

Photodetector device used to measure/detect light scattered off or emitted from particles.

Photomultiplier tube light detector used in flow cytometers and other analytical instruments.

Pia mater thin membrane directly covering the central nervous system; middle layer of the meninges.

Pica perversion of appetite that leads to bizarre eating practices; a clinical finding in some individuals with iron-deficiency anemia.

Pitting removing abnormal inclusions from erythrocytes by the spleen.

PIVKA *See* Protein induced by vitamin K absence or antagonist.

Plasma liquid component of blood.

Plasma cell transformed, fully differentiated B lymphocyte normally found in the bone marrow and medullary cords of lymph nodes. It can be seen in the circulation in certain infections and disorders associated with increased serum γ-globulins. It is characterized by the presence of an eccentric nucleus containing condensed, deeply staining chromatin and deep basophilic cytoplasm. The large Golgi apparatus next to the nucleus does not stain, leaving an obvious clear paranuclear area. The organism has the PC-1 membrane antigen and cytoplasmic immunoglobulin.

Plasma cell neoplasm malignant disorder of the immunoglobulin secreting, fully differentiated B-lymphocyte.

Plasmacytoma localized, tumorous collection of clonal plasma cells. The disease prognosis is related to its location.

Plasma exchange removal of patient plasma and replacement with donor plasma.

Plasmacytoid lymphocyte an intermediate cell in immunoblast development between the B cell and the plasma cell. It has morphologic similarity to the lymphocyte but has marked cytoplasmic basophilia similar to that of plasma cells. It is occasionally seen in the peripheral blood of patients with viral infection.

Plasmacytosis presence of plasma cells in the peripheral blood or an excess of plasma cells in the bone marrow.

Plasmin proteolytic enzyme with trypsin-like specificity that digests fibrin or fibrinogen as well as other coagulation factors; formed from plasminogen.

Plasminogen inactive precursor of the enzyme plasmin; β-globulin, single-chain glycoprotein that circulates in the blood as

a zymogen. Large amounts are absorbed with the fibrin mass during clot formation; it is activated by intrinsic and extrinsic activators to form plasmin.

Plasminogen activator protein of the fibrinolytic system that converts a precursor protein plasminogen to plasmin.

Plasminogen activator inhibitor-1 (PAI-1) primary inhibitorof tissue plasminogen activator (t-PA) and urokinase-like plasminogen activator (tcu-PA) released from platelet α-granules during platelet activation.

Plasminogen activator inhibitor-2 (PAI-2) inhibitor of tissue and urokinase-like plasminogen activator. Its secretion is stimulated by endotoxin and phorbol esters. Increased levels impair fibrinolysis and are associated with thrombosis.

Platelet round or oval disc-shaped structure in the peripheral blood formed from the cytoplasm of megakaryocytes in the bone marrow. It plays an important role in primary hemostasis by adhering to the ruptured blood vessel wall and aggregating to form a plug over the injured area. It is also important in secondary hemostasis by providing phospholipids important for the activation of coagulation proteins. The normal reference range is 150–400 $\times$ 10^9/L.

Platelet activation stimulation of the cell involved in primary hemostasis (platelets) that occurs when agonists bind to its surface and transmit signals to the cell's interior. Activated platelets form aggregates known as the *primary platelet plug.*

Platelet adhesion the ability of the cell involved in primary hemostasis to attach to collagen fibers or other nonplatelet surfaces.

Platelet aggregation the interaction of the cells inolved in primary hemostasis with one another to form a clumped mass; can occur in vitro or in vivo.

Platelet clump aggregation of cells involved in primary hemostasis; can occur when blood is collected by capillary puncture (from cell activation) and when blood is collected in EDTA anticoagulant (the result of unmasking cell antigens that can react with antibodies in the serum).

Platelet distribution width (PDW) coefficient of variation of platelet volume distribution; analogous to RDW.

Platelet factor 4 protein present in the platelet α-granules; is capable of neutralizing heparin.

Plateletpheresis procedure in which cells involved in primary hemostasis are removed from the circulation.

Platelet-poor plasma (PPP) citrated liquid blood component containing $<15 \times 10^9$/L platelets; used for the majority of coagulation tests; prepared by centrifugation of citrated whole blood at a minimum RCF of 1000 $\times$ g for 15 minutes.

Platelet procoagulant activity property of platelet that enables activated coagulation factors and cofactors to adhere to the cell's surface during the formation of fibrin.

Platelet-rich plasma (PRP) citrated blood component containing approximately 200 $\times$ 10^9/L platelets f used in aggregation studies. It is prepared by centrifugation of citrated whole blood at an RCF of 150 $\times$ g for 10 minutes.

Platelet satellitism adherence of pirmary platelets to neutrophil membranes in vitro; can occur when blood is collected in EDTA anticoagulant.

Platelet secretion release of the contents of the platelet's α-granules and dense bodies during platelet activation.

Platelet-type-pseudo VWD disorder characterized by an increased affinity of the platelet GPIb/IX receptor for VWF, resulting in spontaneous binding of the large VWF multimers to the platelet. It resembles VWD clinically and often presents with similar laboratory test results but is not associated with genetic mutations involving the *VWF* gene and thus is not considered "true" VWD.

Pleocytosis abnormally increased number of cells in the cerebrospinal fluid (CSF).

Pleura lining of the cavities between the chest wall and the lungs.

Pleural cavity space between the chest wall and the lungs.

Plethora excess of blood.

Plumbism lead poisoning.

Pluripotential cell cell that differentiates into many different cell lines. It has the potential to self-renew, proliferate, and differentiate into erythrocytic, myelocytic, monocytic, lymphocytic, and megakaryocytic blood cell lineages.

Poikilocytosis presence of erythrocytes with variations in shape.

Point-of-care testing laboratory examination performed at a site close to the patient.

Point-of-care (POC) instrument device designed for analytical testing of patient specimens outside the laboratory setting (e.g., at home or physician's office).

Polychromatophilia quality of being stainable with more than one portion of the stain; commonly used to describe erythrocytes that stain with a grayish or bluish tinge with Romanowsky stains from residual RNA, which takes up the blue portion of the dye.

Polychromatophilic erythrocyte red blood cell with a bluish tinge when stained with Romanowsky stain; contains residual RNA. If stained with new methylene blue, the cell shows reticulum and is identified as a reticulocyte.

Polyclonal derived from different cell clones.

Polyclonal gammopathy alteration in immunoglobulin production characterized by an increase in immunoglobulins of more than one class.

Polycythemia condition associated with increased erythrocyte count.

Polycythemia vera myeloproliferative neoplasm in which there is an increased proliferation of erythroid cells.

Polymerase chain reaction procedure for copying a specific DNA sequence many times.

Polymorphic being genetically variable.

Polymorphic variant version of the form and structure of a portion of a chromosome that has no clinical consequence.

Polymorphism condition in which alternate copies (alleles) of a gene are present.

Polymorphonuclear having the form and structure of the nucleus of the granulocyte in which the nucleus is segmented.

Polymorphonuclear neutrophil (PMN) mature granulocyte found in bone marrow and peripheral blood that primarily functions as defense against foreign antigens. The nucleus is segmented into two or more lobes. The cytoplasm stains pinkish, and specific granules are abundant. This is the most numerous leukocyte in the peripheral blood ($1.8–7.0 \times 10^9$/L) and is active in phagocytosis and killing microorganisms. Also called *segmented neutrophil* or *seg.*

Polyploid having a number of chromosomes per cell that is a multiple of *n* (23) other than 1n or 2n (e.g., 3n[69], 4n[92]).

Polyploidy pertaining to cells and organisms that contain more than two paired (homologous) sets of chromosomes.

Popcorn cell (L&H cell) neoplastic cell found in LP Hodgkin lymphoma characterized by a delicate multilobated nucleus and multiple, small nucleoli. It has a B cell phenotype: LCA+ (leukocyte common antigen), CD20+, CD15.

Porphyria group of inherited disorders characterized by a block in porphyrin synthesis caused by a defect in one or more of the enzymes in the heme synthesis pathway. It causes a buildup of compounds of heme precursors that accumulate in tissues and are excreted in large amounts in the urine and/or feces. The two forms are hepatic and erythropoietic. Only the erythropoietic types affect the erythrocytes.

Porphyrin any of various compounds of highly unsaturated tetrapyrrole ring bonded by 4 methane (−CH =) bridges; is metabolically active only when chelated. Substituents occupy each of the eight peripheral positions on the 4-pyrrole rings. The type and order of these substituents determine the type of compound.

Portland hemoglobin embryonic intracellular erythrocyte protein in the yolk sac that is detectable up to 8 weeks of gestation. It is composed of two zeta (ζ) and two gamma (γ) chains.

Postmitotic pool compartment of neutrophils including metamyelocytes, bands, and segmented ones in the bone marrow that are not capable of mitosis. Cells spend about 5–7 days in this compartment before being released to the peripheral blood. Also called *maturation-storage pool.*

Post-translational modification process occurring in eukaryotic cells that change the protein product resulting from the ribosomal translation; it can involve the addition of sugar groups (glycosylation) and phosphate groups (phosphorylation) or other modifications to amino acids (e.g., gamma carboxylation of coagulation proteins).

Precursor neoplasm acute abnormal tissue derived from a mutated precursor cell and represented by an increased number of blasts in the peripheral blood and bone marrow.

Primary aggregation earliest association of platelets in a reversible combination.

Primary fibrinogenolysis clinical situation that occurs when excessive quantities of plasminogen activators are released into the blood in the absence of fibrin clot formation. Excess plasmin degrades fibrinogen and the clotting factors, leading to a potentially dangerous hemorrhagic condition.

Primary hemostasis initial arrest of bleeding that occurs with blood vessel–platelet interaction.

Primary hemostatic plug platelet aggregate that initially halts blood flow from an injured vessel.

Primary myelofibrosis neoplastic clonal hematopoietic stem cell disorder with splenomegaly, leukoerythroblastosis, extramedullary hematopoiesis (myeloid metaplasia), and progressive bone marrow fibrosis; classified as a myeloproliferative neoplasm (WHO 2008). Also known as *myelofibrosis with myeloid metaplasia.*

Primary thrombocytosis disease caused by an increase in platelets that is not secondary to another condition. It usually refers to the increase in platelets that can occur in neoplastic disorders.

Probe tool for identifying a particular nucleotide sequence of interest. It is composed of a nucleotide sequence that is complementary to the sequence of interest and is therefore capable of hybridizing to the sequence. Probes are labeled in a way that is detectable, such as by radioactivity.

Procoagulant inert precursor of a natural substance necessary for blood clotting or a property of anything that favors formation of a blood clot.

Proficiency testing examining unknown samples from an external source (e.g., College of American Pathologists) to monitor the quality of a given laboratory's test results.

Progenitor cell parent or ancestor cell that differentiates into mature, functional cells.

Prolymphocyte immediate precursor of the lymphocyte; normally found in bone marrow. It is slightly smaller than the lymphoblast and has a lower nuclear-to-cytoplasmic ratio. The nuclear chromatin is somewhat clumped, and nucleoli are usually present. The cytoplasm stains light blue and is agranular.

Promonocyte monocytic precursor cell found in the bone marrow; is 14–18 mcM (μm) in diameter with abundant blue-gray cytoplasm. Fine azurophilic granules can be present. The nucleus is often irregular and deeply indented. The chromatin is finely dispersed and stains a light purple blue. Nucleoli can be present. Cytochemically, the cells stain positive for nonspecific esterase, peroxidase, acid phosphatase, and arylsulfatase. The cell matures to a monocyte.

Promyelocyte granulocytic precursor cell normally found in the bone marrow; is 15–21 mcM (μm) in diameter. The cytoplasm is basophilic, and the nucleus is quite large. The nuclear chromatin is lacy, staining a light purple blue. Several nucleoli are visible. The distinguishing feature is the presence of large blue-black primary (azurophilic) granules that have a phospholipid membrane that stains with Sudan black B. The granules contain acid phosphatase, myeloperoxidase, acid hydrolases, lysozyme, sulfated mucopolysaccharides, and other basic proteins. The cell matures to a myelocyte. Also called *progranulocyte.*

Pronormoblast precursor cell of the erythrocyte. The cell is derived from the pluripotential stem cell and is found in the bone marrow. The cell is 12–20 mcM (μm) in diameter and has a high nuclear-cytoplasmic ratio. The cytoplasm is deeply basophilic with

Romanowsky stains. The nuclear chromatin is fine and has one or more nucleoli. This cell matures to a basophilic normoblast. Also called *rubriblast*.

Proplatelet group of platelets comprising a long, slender protrusion of megakaryocyte cytoplasm released from mature megakaryocytes. It breaks up into individual platelets.

Protein induced by vitamin K absence or antagonists factor that is the nonfunctional form of the prothrombin group of coagulation factors. It is synthesized in the liver in the absence of vitamin K and lacks the carboxyl (COOH) group necessary for binding the factor to a phospholipid surface (*acarboxy* form).

Proteomics study of the structure and function of proteins in a cell or tissue at a specific time under certain predefined conditions; includes information on how the proteins interact with each other inside cells.

Proteosome eukaryotic assembly of proteins that degrades other proteins.

Prothrombinase complex complex formed by coagulation factors Xa and V, calcium, and phospholipid; activates prothrombin to thrombin.

Prothrombin group assemblage of coagulation factors that are vitamin K dependent for synthesis of their functional forms and that require calcium for binding to a phospholipid surface. It includes factors II, VII, IX, and X. Also known as *vitamin K–dependent factor*.

Prothrombin time (PT) screening test used to detect deficiencies in the extrinsic and common pathway of the coagulation cascade and to monitor the effectiveness of oral anticoagulant therapy.

Prothrombin time ratio proportion calculation derived by dividing the patient's prothrombin time result by midpoint of the laboratory's normal range and to calculate the International Normalized Ratio (INR).

Proto-oncogene normal hereditary unit that has the potential to cause cancer when it mutates to become a dominant-acting oncogene. It is normally involved in regulating the cell cycle, cell differentiation and maturation, and apoptosis.

Prourokinase immature, single-chain form of enzyme prepared from urine and by recombinant DNA techniques; can be activated to a two-chain form by plasmin.

Pseudochylous fluid that appears chylous because of the presence of many inflammatory cells; does not contain lymph fluid or chylomicrons.

Pseudodiploid cell that has a chromosome count of $2n$ (46) but with a combination of numerical and/or structural aberrations (e.g., 46, XY, −5, −7, 2D8, 2D21).

Pseudo-neutrophilia increase in the concentration of neutrophils in the peripheral blood ($>7.0 \times 10^9$/L) occurring as a result of cells from the marginating pool entering the circulating pool. The response is immediate but transient. This redistribution of cells accompanies vigorous exercise, epinephrine administration, anesthesia, convulsion, and anxiety states. Also called *immediate* or *shift neutrophilia*.

Pseudo–Pelger-Huët cell unusually acquired condition in which neutrophils display a hyposegmented nucleus. Unlike the real Pelger-Huët anomaly, this cell's nucleus contains a significant amount of euchromatin and stains more lightly. A critical differentiation point is that all neutrophils are equally affected in the genetic form of Pelger-Huët anomaly, but only a fraction are hyposegmented cells in the acquired state. It is associated with MDS and MPD and can also be found after treatment for leukemias.

Pulmonary embolism obstruction of the pulmonary artery or one of its branches by a clot or foreign material that has been dislodged from another area by the blood current.

Pure red cell aplasia (PRCA) anemia with selective decrease in erythrocyte precursors in the marrow.

Purging cleansing that removes undesirable cells that are present in the blood or bone marrow products.

Purpura (1) purple discoloration of the skin caused by petechiae and/or ecchymoses; (2) diverse group of disorders characterized by the presence of petechiae and ecchymoses.

Pyknotic pertaining to degeneration of the cell's nucleus in which the chromatin condenses to a solid, structureless mass and shrinks.

Quality control limit expected range of results used to determine whether a test method is in control and to minimize the chance of inaccurate results. If the test method is out of control, an intervention is required to reconcile the inaccuracy.

Quebec platelet disorder storage pool abnormality of platelets caused by aberrant proteolysis of α-granule proteins resulting from increased levels of urinary-type plasminogen activator.

Quiescence (G_0) phase in a cell that has exited the cell cycle and is in a nonproliferative state.

Relaxed (R) structure conformational change in hemoglobin that occurs as the molecule takes up oxygen.

Radar chart graphical representation of eight CBC parameters: WBC, RBC, Hb, Hct, MCV, MCH, MCHC, and PLT. Lines are drawn to connect these parameters; representation resembles a radar oscilloscope. Changes in the shape of the radar chart indicate different hematologic disorders.

Radial immunodiffusion technique in which an antibody is incorporated into an agarose gel into whose wells the antigen is placed. The antigen is quantitated by the size of a precipitin ring that forms as the antigen diffuses from a sample well into the gel.

Random access capability of an automated hematology instrument to process specimens independently of one another; can be programmed to run individual tests (e.g., Hb or platelet counts) or a panel of tests (e.g., CBC with reticulocyte count) without operator intervention.

Random variation fluctuation within an instrument or test method caused by chance. It can be either positive or negative in direction and affects precision.

Rapoport-Luebering shunt metabolic pathway in which 2,3-bisphosphoglycerate (2,3-BPG) is synthesized from 1,3-bisphosphoglycerate. 2,3-BPG facilitates the release of oxygen from hemoglobin in the erythrocyte. Also referred to as *2,3-diphosphoglycerate* (*DPG*).

Raynaud's phenomenon secondary disorder resulting from vaso-arterial spasms in the extremities of the body when exposed

to the cold. It is characterized by blanching of the skin followed by cyanosis and finally redness when the affected area is warmed. Also referred to as *acrocyanosis.*

RBC indices measurements that help classify erythrocytes as to their size and hemoglobin content. The values for hemoglobin, hematocrit, and erythrocyte are used to calculate the three indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH). They give a clue as to what the erythrocytes should look like on a stained blood film.

Reactive lymphocytosis self-limiting, reactive process resulting in an increase in lymphocytes that occurs in response to an infection or inflammatory condition. Both T and B types are commonly affected, but their function remains normal.

Recombination-activating genes 1 and 2 (*RAG-1, RAG-2*) enzyme components of the V(D)J recombinase complex expressed in developing B and T cells, which are critical for DNA recombination events that form functional immunoglobulin and T-cell receptor genes.

Red blood cell (RBC) cellular element of blood that carries the protein hemoglobin. Its function is to carry oxygen and carbon dioxide between the lungs and the tissues. It does not have a nucleus and develops in the bone marrow from the hematopoietic stem cell. When released to the peripheral blood, it has a lifespan of about 120 days. Also called an *erythrocyte*.

Reactive lymphocyte antigen-stimulated white blood cell that exhibits a variety of morphologic features. It is usually larger and has a more basophilic cytoplasm than the resting cell and has an irregular shape. Its nucleus is often elongated and irregular with a finer chromatin pattern than that of the resting lymphocyte. Often this cell is increased in viral infections. Also called *virocyte,* or *stimulated, transformed, atypical, activated,* or *leukocytoid lymphocyte.*

Reactive neutrophilia increase in the concentration of peripheral blood neutrophils ($>7.0 \times 10^9$/L) as a result of reaction to a physiologic or pathologic process.

Reagent blank measurement of absorbance from a substance alone that eliminates a false increase in sample absorbance caused by the substance's color.

Recombinant factor VIIa (rFVIIa) form of blood factor VII that has been manufactured via laboratory method of genetic technology.

Red cell distribution width (RDW) coefficient of variation of the MCV; a calculated index provided by hematology analyzers to help identify anisocytosis (standard deviation of MCV $\times$ 100/mean MCV) with a reference interval of 11.5–14.5%.

Red thrombus blood clot formed in the veins where flow is slower than in other areas**;** primarily composed of red blood cells trapped in the fibrin mesh with relatively few platelets and leukocytes.

Reed-Sternberg (R-S) cell cell found in the classic form of Hodgkin lymphoma; characterized by a multilobated nucleus and large inclusion-like nucleoli.

Reference interval test value range considered to be normal and generally determined to include 95% of the normal population.

Reflex testing follow-up examination performed as the results of screening tests.

Refractive index degree to which a transparent object will deflect a light ray from a straight path.

Refractory pertaining to disorders or diseases that do not respond readily to therapy.

Refractory anemia subgroup of the WHO 2008 classification of the myelodysplastic syndrome: Refractory cytopenia with unilineage dysplasia nonresponse to all conventional therapy is the primary clinical finding. Blasts constitute $<1\%$.

Refractory anemia with excess blasts-1 (RAEB-1) subgroup of WHO 2008 classification of myelodysplastic syndromes. There are usually cytopenias and signs of dyspoiesis in the peripheral blood with $<5\%$ blasts.

Refractory anemia with excess blasts-2 (RAEB-2) category in the WHO 2008 classifcation systemof the myelodysplastic syndromes. Cytopenia in the peripheral blood has 5%–19% blasts. The bone marrow is usually hypercellular with dyspoiesis and 10–19% blasts. The presence of Auer rods in the blasts qualifies a diagnosis as RAEB-2 regardless of the blast count ($>20\%$ blasts).

Refractory anemia with ring sideroblasts (RARS) category in the WHO 2008 classification system; of myelodysplastic syndromes characterized by $<1\%$ blasts in the peripheral blood, anemia, and/or thrombocytopenia and/or leukopenia. Bone marrow has $>15\%$ ring sideroblasts and $<5\%$ blasts.

Refractory cytopenia with multilineage dysplasia (RCMD) category in the WHO 2008 classification system of the myelodysplastic syndromes; characterized by dysplastic features in at least 10% of the cells in two or more cell lines, $<5\%$ blasts in the bone marrow, and $<1\%$ blast in the peripheral blood.

Refractory cytopenia with unilineage dysplasia category in the 2008 WHO classification of the myelodysplastic syndromes; characterized by isolated decrease in one or two cell lines accompanied by unilineage dysplasia. It includes anemia that is unresponsive to treatment and less common cases of neutropenia and thrombocytopenia that are unresponsive to treatment.

Regulatory T lymphocyte (T_{Reg}) subpopulation of T lymphocytes that suppress the immune response and maintain peripheral tolerance to self-antigens (i.e., suppress autoimmunity); this subpopulation primarily includes CD4+, CD25+, FoxP-3+.

Remission diminution of disease symptoms.

Replication process by which DNA is copied during cell division by the enzyme DNA polymerase, which recognizes single-stranded DNA and fills in the appropriate complementary nucleotides to produce double-stranded DNA. Synthesis is initiated at a free 5′ end where double-stranded DNA lies adjacent to single-stranded DNA, and replication proceeds in the 5′ direction. In the laboratory, the DNA duplication can be induced as a means of copying DNA sequences as exploited in the polymerase chain reaction.

Reportable range span defined by a minimum value and a maximum value of calibration material.

Reticulated platelet cell involved in primary hemostasis that is newly released from the bone marrow; possesses residual RNA.

Restriction endonuclease enzyme that cleaves double-stranded DNA at specific nucleotide sequences. For example, HindIII cleaves DNA only where the sequence 5′-AAGCTT-3′ is present. Various other enzymes are known to cut some specific target sequences. Examples of common ones are BamH1, EcoR1, Mnl1, MstII, Pst1, and Xba1.

Restriction point stage that occurs in late G_1 that occurs when cell cycle progression becomes autonomous.

Reticular cell one of the three major types of cells in the bone marrow stroma. It also is found in the spleen and lymph nodes. These cells branch to form reticular fibers. Also called *fibroblasts*.

Reticulocyte first non-nucleated stage of erythrocyte development in the bone marrow. It contains RNA that is visualized as granules or filaments within the cell when stained supravitally with new methylene blue. Normally, this stage constitutes approximately 1% of the circulating erythrocyte population.

Reticulocyte hemoglobin (RET-He) reticulocyte hemoglobin reported by the Sysmex automated hematology analyzers; analogous to the MCH of mature erythrocytes.

Reticulocyte production index (RPI) a calculated value that is an indicator of bone marrow response in anemia. Its calculation corrects the reticulocyte count for the presence of marrow reticulocytes in the peripheral blood. It is calculated as follows:

RPI = (Patient hematocrit [L/L] ÷ 0.45 [L/L] × reticulocyte count [%]) × (1 ÷ maturation rime of shift reticulocytes)

Reticulocytosis presence of excess reticulocytes in the peripheral blood.

Rh null disease disorder associated with the lack of the Rh antigen on erythrocytes.

Rhopheocytosis process in which ferritin molecules are endocytosed on erythroblasts; a form of micropinocytosis.

Ribosomes cellular particle composed of ribonucleic acid (RNA) and protein whose function is to synthesize polypeptide chains from amino acids. The sequence of amino acids in the chains is specified by the genetic code of messenger RNA. This particle appears singly or in reversibly dissociable units and can be free in the cytoplasm or attached to endoplasmic reticulum. The cytoplasm of blood cells that contain a high concentration of ribosomes stains bluish purple with Romanowsky stains.

Richter's transformation change from CLL to another disease, usually large B-cell lymphoma.

Ring sideroblast erythroblast with abnormal deposition of excess iron within mitochondria resulting in a ring formation around the nucleus.

Ristocetin aggregating reagent that specifically evaluates VWF interaction with glycoprotein Ib on platelets.

Ristocetin cofactor property of the aggregating reagent when it acts as a cofactor in the ristocetin cofactor assay that screens for binding of VWF to platelet GPIb. When this reagent is added to a suspension of platelets in plasma that contains VWF, it promotes the binding of VWF to platelet membrane GPIb, causing platelet aggregation at a rate depending on the concentration of VWF and reagent.

Ristocetin-induced platelet aggregation (RIPA) measures ability of patient's VWF to bind to normal platelets, inducing platelet combining in a mass in a platelet aggregation assay.

RNA (ribonucleic acid) single-stranded molecule composed of ribonucleotides (A, C, G, and U) produced by transcription of genes from a DNA template. This molecule in turn serves as a template for protein translation.

Romanowsky-type stain any dye consisting of methylene blue and its oxidation products and eosin Y or eosin B. Wright's stain, a Romanowsky-type stain, is commonly used to stain hematopoietic cells.

Rouleaux erythrocyte distribution characterized by being stacked like a roll of coins. This is the result of abnormal coating of the cell's surface with increased plasma proteins, decreasing the zeta potential between cells.

Rule of three in general, the formula is hemoglobin × 3 = hematocrit that is used as a quick mathematical check on the accuracy of the measurement of hemoglobin and hematocrit. It works only with normochromic, normocytic erythrocytes. If the calculated hematocrit does not agree within ± 3% of the measured hematocrit, investigation for an error or instrument malfunction should be conducted. The patient also can have a disorder that needs further investigation.

Russell body globule filled with immunoglobulin found in pathologic plasma cells called *Mott cells*. *See* Mott cell.

Russell's viper venom poisonous substance that possesses thromboplastin-like activity and activates factor X.

Satellite DNA nucleic acid containing many tandem repeats. Morphologically, it appears as a small ball-like structure making up the short arm of acrocentric chromosomes. It is the locus of the nucleolar organizing region.

Scatterplot dot-plot histogram of two cellular characteristics. Together, the two characteristics allow definition of the leukocyte subpopulations.

Schilling test definitive analysis used in distinguishing cobalamin (vitamin B_{12}) deficiency caused by malabsorption, dietary deficiency, or absence of IF. It measures the amount of an oral dose of radioactively labeled crystalline vitamin B_{12} that is absorbed in the gut and excreted in the urine.

Schistocyte fragment of an erythrocyte that can have a variety of shapes including triangle, helmet, and comma.

Scott syndrome rare platelet disorder characterized by abnormal Ca^{++} induced phospholipids scrambling in which platelet membranes fail to support plasma procoagulant protein activation.

Secondary aggregation irreversible mass of platelets that occurs over time.

Sea blue histiocytosis syndrome rare inherited disorder characterized by splenomegaly and thrombocytopenia. Sea blue-staining macrophages are found in the liver, spleen, and bone marrow. The cell is large (in diameter) with a dense eccentric nucleus and cytoplasm that contains blue or blue-green granules. In most patients, the course of the disease is benign.

Secondary fibrinolysis clinical condition characterized by excessive fibrinolytic activity in response to disseminated intravascular clotting.

Secondary hemostasis formation of fibrin that stabilizes a primary platelet plug.

Secondary hemostatic plug primary platelet aggregate that has been stabilized by fibrin formation during secondary hemostasis.

Secondary (reactive) thrombocytosis disorder in which platelet concentration in the blood increases in response to stimulation by another condition.

Secretion energy-dependent discharge or release of products usually from glands in the body after stimulation of the platelets by agonists; also the product that is discharged or released.

Self-renewal property of regenerating the same cells.

Semen the fluid in the male that contains spermatozoa and is discharged at ejaculation.

Sequestration crisis sudden splenic pooling of sickled erythrocytes that can cause a large decrease in erythrocyte mass within a few hours.

Serine protease family of enzymes that cleave peptide bonds in proteins including thrombin, factors VIIa, IXa, Xa, XIa, XIIa, and the digestive enzymes chymotrypsin and trypsin. It selectively hydrolyzes arginine- or lysine-containing peptide bonds of other zymogens, converting them to serine proteases. Each involved in the coagulation cascade is highly specific for its substrate.

Serpin family of proteins with similar structures that inhibit target molecules by forming a 1:1 stoichiometric complex.

Serum transferrin receptor (sTfR) extracellular portion of the protein of the β-globulin group receiver that is cleaved by proteolytic enzymes as the erythrocytes mature and is released into the blood. When erythropoiesis increases, the sTfR increase.

Severe combined immunodeficiency syndrome (SCIDS) most severe form of a heterogeneous group of disorders in which there is a lack of ability to mount an immune response; The disease can be inherited either as a sex-linked trait or as an autosomal-recessive trait.

Sézary cell circulating neoplastic cell found in patients with Sézary syndrome; it is a malignant mature memory helper T lymphocyte which is characterized by a very convoluted (cerebriform) nuclear outline.

Shelf life time period for which a reagent or control is stable given appropriate storage conditions but changes once the reagent or control is reconstituted if lypholyzed or opened if liquid.

Shift neutrophilia *See* Pseudo-neutrophilia.

Shift to the left (left shift) appearance of increased numbers of immature leukocytes in the peripheral blood.

Sickle cell (drepanocyte) elongated crescent-shaped erythrocyte with pointed ends whose formation may be observed in wet preparations or in stained blood smears from patients with sickle cell anemia. These cells are formed when the HbS becomes deoxygenated and polymerizes into rigid aggregates.

Sickle cell anemia genetically determined disorder in which hemoglobin S is inherited in the homozygous state. ($\beta^s\beta^s$) No hemoglobin A is present. Hemoglobins S, F, and A_2 are present.

Sickle cell trait genetically determined disorder in which hemoglobin S is inherited in the heterozygous state. The patient has one normal β-globin gene and one β^s-globin gene. Both hemoglobins A and S are present as well as hemoglobins F and A_2.

Side light scatter laser light distributed at a 90° angle caused by a particle's internal complexity and granularity (e.g., neutrophils produce much side spread because of their numerous cytoplasmic granules).

Sideroblast erythroblast that contains stainable iron granules.

Sideroblastic anemia form of congenital and acquired disorders characterized by an increase in total body iron, the presence of ring sideroblasts in the bone marrow, hypochromic erythrocytes in the peripheral blood, and deficiency of hemoglobin and/or erythrocytes. The acquired idiopathic form is classified as a myelodysplastic syndrome (MDS).

Siderocyte erythrocyte that contains stainable iron granules.

Sideropenic lack of iron.

Sideropenic anemia blood disorder caused by insufficient iron for erythropoiesis resulting in a deficiency of hemoglobin.

Single nucleotide polymorphism (SNP) change in which a single base in the DNA differs from the usual base at that position.

Slope angle or direction of the regression line with respect to the x- and y-axes that is used to identify the presence of proportional systematic error.

Small lymphocytic lymphoma (SLL) condition identical to CLL except that it primarily involves the lymph nodes instead of the bone marrow and peripheral blood. The two disorders appear to belong to one disease entity with differing clinical manifestations.

Smooth endoplasmic reticulum (SER) *See* Endoplasmic reticulum.

Smudge cell leukocyte whose cytoplasmic membrane has ruptured, leaving a bare nucleus. Increased numbers are observed in lymphoproliferative disorders such as chronic lymphocytic leukemia. The ruptured leukocyte can also be seen in reactive lymphocytosis and in other neoplasms. An increased number of these leukocytes can result in a falsely decreased WBC count and erroneous differential.

Southern blot procedure first described by Ed Southern for determining DNA structure. In it, DNA is cleaved with restriction endonucleases that cut DNA at specific nucleotide sequences. The resulting DNA fragments are electrophoresed in an agarose gel to separate them by size and are then treated with a solution of high pH that separates double-stranded DNA into two single-stranded parts. The single-stranded fragments are then transferred to a membrane where they can be hybridized to a complementary labeled probe. Probe hybridization permits identification of the DNA fragments containing the sequence of interest. The size and number of those fragments reflects the structure of the DNA.

Specific esterase enzyme that hydrolyzes particular esters; can be detected in cells with a stain using naphthol AS-D chloroacetate at an acid pH; is considered specific for the granulocytic series, and is present in mast cells; used to differentiate monoblasts and myeloblasts

Specificity ability of a test method to determine only the analyte meant to be detected or measured.

Specimen run interval, period of time, or number of specimens for which the accuracy and precision of the laboratory procedure is expected to remain stable.

Spectrin predominant peripheral membrane protein composed of dimeric chains, α and β, that associate to form tetramers; found in the erythrocyte cytoskeleton.

Spent phase stage in polycythemia vera in which after a period of 2–10 years, the patient can develop bone marrow failure accompanied by an increase in splenomegaly. Anemia and bleeding can be the primary clinical findings, secondary to a decreased platelet count and decreasing hematocrit. This phase is often a transition to AML.

Sperm male gamete formed in the seminiferous tubules. Also known as *spermatozoa.*

Spherocyte abnormally round erythrocyte with dense hemoglobin content (increased MCHC) and no central area of pallor because it has lost its biconcave shape.

Splenectomy removal of the spleen.

Splenomegaly abnormal enlargement of the spleen.

Split sample division of a single specimen into two or more aliquots for testing that uses two or more instruments within the same time period or retesting the sample at another time.

Spur cell anemia acquired hemolytic condition associated with severe hepatocellular disease such as cirrhosis in which serum lipoproteins increase, leading to an excess of erythrocyte membrane cholesterol. The total phospholipid content of the membrane, however, is normal. The predominant poikilocyte is an erythrocyte with irregular points and no area of central pallor (acanthocytes).

Stab *See* Band neutrophil.

Stage phase of a neoplastic disease defined by the extent and distribution of disease. Determining the phase of disease usually involves radiologic studies, peripheral blood examination, bone marrow aspiration, and biopsy.

Standard deviation distribution of a set of data about the mean.

Standard error of the estimate ($S_{y/x}$) measure of the variation in the regression line. The $S_{y/x}$ is used to identify random error.

Starry sky appearance morphologic pattern characteristic of high-grade lymphoma produced by numerous tingible body macrophages (stars) and a diffuse sheet of neoplastic cells (sky).

Stomatocyte abnormal erythrocyte shape characterized by a slit-like area of central pallor and a uniconcave, cup shape.

Streptokinase bacterial enzyme derived from group C-beta hemolytic streptococci; the enzyme activates plasminogen to plasmin and is used as a thrombolytic agent in the treatment of thrombosis.

Stroma extracellular matrix or microenvironment that supports hematopoietic cell proliferation in the bone marrow.

Stromal cell element of the hematopoietic microenvironment in the red portion of bone marrow.

Submetacentric chromosome that has the centromere positioned off center so that one arm is shorter than the other arm.

Sucrose hemolysis test screening examination to identify erythrocytes that are abnormally sensitive to complement lysis. When erythrocytes, serum, and sucrose are incubated together, cells abnormally sensitive to complement lyse. The test is used to screen for paroxysmal nocturnal hemoglobinuria. Also called *sugar-water test*.

Sulfhemoglobin stable compound formed when a sulfur atom combines with each of the four heme groups of the intracellular erythrocyte oxygen carrying protein; when this protein is combined with sulfur, it is incapable of carrying oxygen.

Supernatant clear liquid remaining on top of a solution after centrifugation of the particulate matter.

Supravital stain agent used to color cells or tissues while they are still living.

Syngeneic in transplantation biology, having identical genotypes.

Syngeneic stem cell transplantation medical process of moving stem cells between genetically identical twins.

Synovium continuous membrane that lines the bony, cartilaginous, and connective tissue surfaces of a joint.

Systemic mastocytosis abnormal clonal proliferation of mast cells in at least one extracutaneous organ with or without evidence of skin lesions; the bone marrow is almost always involved but the peripheral blood rarely has significant numbers of circulating mast cells.; laboratory studies that demonstrate a mutation of the *c-Kit* proto-oncogene and an aberrant immunophenotype are needed to make the diagnosis. .

Systematic variation difference within an instrument or test method that occurs in one direction, can be predicted, and affects accuracy.

T_H17 lymphocytes subpopulation of CD4+ T cells identified for their production of IL-17, this subpopulation of cells induce an inflammatory response and aid in host defense against extracellular pathogens.

Target cell abnormally shaped erythrocyte that has decreased osmotic fragility and appears as a target with a bull's-eye center mass of hemoglobin surrounded by an achromic ring and an outer ring of hemoglobin. Also called *Mexican hat cell* and *codocyte.*

Tartrate-resistant acid phosphatase (TRAP) an acid phosphatase whose activity is not inhibited following tartrate incubation; found in hairy cells of hairy cell leukemia.

T-cell acute lymphoblastic leukemia (ALL) neoplasm of the white blood cells, and immunologic subgroup of ALL. The neoplastic cells are precursor cells of the lymphocytic lineage with T-cell antigens.

T-cell receptor (TCR) antigen receiver on immunocompetent T lymphocytes.

Teardrop (dacryocytes) erythrocyte that is elongated at one end to form a teardrop or pear-shaped cell; can form after erythrocytes with cellular inclusions have transversed the spleen. The erythrocyte cannot return to its original shape because it has either been stretched beyond its limits of deformability or has been in the abnormal shape too long.

Telangiectasia condition characterized by persistent dilation of superficially located veins.

Tense (T) structure conformational change in the makeup of a hemoglobin molecule that occurs when oxygen is released from hemoglobin.

Terminal deoxynucleotidyl transferase (TdT) DNA polymerase found in pre-B and pre-T lymphoid precursor cells, which adds nucleotides to V, D, and J segments during DNA recombination events that form functional immunoglobulin and T-cell receptor genes; is responsible for junctional diversity.

T_H17 cell See T_H17 lymphocyte.

Thalassemia group of genetically determined microcytic, hypochromic anemias resulting from a decrease in synthesis of one or more globin chains in the hemoglobin molecule. The disorder can occur in the homozygous or heterozygous state. Heterozygotes can be asymptomatic or have a mild disease, but homozygotes typically have a severe, often fatal, disease. Thalassemia occurs most frequently in populations from the Mediterranean area and Southeast Asia.

Therapeutic range level of a drug that is beneficial but not toxic to the individual.

Threshold limit level above which voltage pulses of particles are counted on an automated hematology analyzer. Adjusting the threshold limit allows different types of cells to be counted.

Thrombin activatable fibrinolysis inhibitor (TAFI) protein that inhibits fibrinolysis when activated by the thrombin-thrombomodulin complex.

Thrombocyte *See* Platelet.

Thrombocytopenia deficiency of platelets in the blood; decrease in the number of platelets in the peripheral blood below the reference range for an individual laboratory (usually $<150 \times 10^9$/L).

Thrombocytopenia with absent radii (TAR) inherited condition characterized by isolated hypoplasia of the megakaryocytic lineage, thrombocytopenia, and bilateral radial aplasial.

Thrombocytosis disorder in which the body produces too many platelets (thrombocytes) that play an important role in blood clotting; the number of platelets in the peripheral blood is above the reference range for an individual laboratory (usually $>400 \times 10^9$/L).

Thromboembolism blockage of a small blood vessel by a blood clot that was formed in the heart, arteries, or veins and dislodged and moved through blood vessels until reaching a smaller vessel and blocking further blood flow.

Thrombogenic tendency to clot; to promote the formation of a clot.

Thrombolytic therapy treatment designed to dissolve or break down a blood clot.

Thrombomodulin integral membrane glycoprotein found on endothelial cells that forms a 1:1 complex with thrombin; named for its ability to alter the activity of the proteolytic enzyme thrombin from procoagulant to anticoagulant so that it loses its ability to cleave fibrinogen to fibrin and to activate FV, FVIII, or platelets but instead rapidly activates PC in the presence of Ca^{++}.

Thrombophilia tendency to form blood clots abnormally. Also referred to as *hypercoagulability*.

Thrombophlebitis thrombosis within a vein that is accompanied by an inflammatory response, pain, and redness of the area.

Thrombopoietin cytokine that regulates the maturation of megakaryocytes and the production of platelets.

Thrombosis formation or presence of a clot, usually considered to be under abnormal conditions within a blood vessel.

Thrombotic thrombocytopenic purpura (TTP) acute disorder characterized by microangiopathic anemia, decreased number of platelets, and renal failure as well as neurological symptoms. It is caused by decreased activity of ADAMTS-13, resulting in the presence of ultralarge VWF molecules in circulation and platelet agglutination.

Thrombus blood clot within the vascular system.

TIBC *See* Total iron-binding capacity.

Tingible body macrophage tissue cell of the immune system phagocytosing, engulfing, and usually destroying fragments of dying cells. It is found in areas of extensive apoptosis (reactive germinal centers and high-grade lymphoma).

Tissue factor coagulation component present on subvascular cells that forms a complex with factor VII when the vessel is ruptured. This complex activates factor X and is an integral protein of the cell membrane.

Tissue factor pathway inhibitor (TFPI) intrinsic sequence that restrains both FVIIa and FXa by forming a quarternary complex of TFPI-FXa-TF-FVIIa.

Tissue homeostasis maintenance of an adequate number of cells to carry out the organism's functions. It is controlled by cell proliferation, cell differentiation, and cell death (apotosis).

Tissue-plasminogen activator (tPA) serine protease that activates plasminogen to plasmin. It forms a bimolecular complex with fibrin, increasing the catalytic efficiency of tPA for plasminogen activation.

T lymphoid cell antigen CD marker that when present is unique to and useful in differentiating a T cell from a B cell; used in differentiating TALL from other types of acute leukemia.

Total iron-binding capacity (TIBC) Maximum amount of iron able to be bound in the serum; is measured by transferrin. Each gram of transferrin binds 1.4 mg of iron. Enough transferrin is present in plasma to bind 253–435 mcg (μg) of iron per deciliter of plasma.

Toxic granule large, dark blue-black primary particle in the cytoplasm of neutrophils present in certain infectious states; usually seen in conjunction with Döhle bodies.

Toxoplasmosis condition that results from infection with *Toxoplasma gondii*; can be asymptomatic or symptoms can resemble infectious mononucleosis. It is characterized by a leukocytosis with relative lymphocytosis or rarely an absolute lymphocytosis and the presence of reactive lymphocytes.

Trabecula projection of calcified bone extending from cortical bone into the marrow space; provides support for marrow cells.

Transcription synthesis of RNA from a DNA template.

Transcription error mistake made in reporting test results when copying results manually from the instrument to a paper or electronically by typing them into a computer.

Transcription factor (TF) protein that controls the switching on or off the transcription of genes; It binds to regulatory regions in the genome and helps control gene expression.

Transferrin (Tf) plasma β_1-globulin responsible for binding iron and transporting it in the bloodstream. Each gram can bind 1.25 mg of iron. The capacity of transferrin to bind iron is functionally measured as the total iron-binding capacity (TIBC).

Transferrin receptor 1 (TfR1) transmembrane glycoprotein dimer with two identical subunits, each of which can bind a molecule of transferrin; present on virtually all cells but found in high numbers on cells with high iron requirements.

Transferrin receptor 2 (TfR2) transmembrane glycoprotein found predominantly on hepatocytes, duodenal crypt cells, and erythroid cells; it interacts with HFE to regulate hepcidin synthesis and thus has a role in regulating total body iron homeostasis.

Transferrin saturation amount of plasma β_1-globulin that is complexed with iron. Calculated by serum iron/TIBC $\times$ 100 = % saturation; is normally about one-third saturated with iron.

Transglutaminase the activity of clotting FXIII that promotes the formation of stable covalent cross-links between glutamine and lysine residues on strands of fibrin; FXIIIa is the only coagulation protein with this activity.

Transient erythroblastemia of childhood (TEC) temporary suppression of red blood production that frequently occurs after a viral infection in infants and children. Therapy is supportive, and patients usually recover within 2 months.

Translation step in protein biosynthesis in which synthesis of a protein from an RNA template occurs.

Translocation abnormal chromosomal rearrangement in which part of one chromosome breaks off and becomes attached to another one. The site of juxtaposition between the two chromosomes is referred to as the *break point*.

Transplancental idiopathic thrombocytopenic purpura decrease in platelets that is present in newborns because of maternal transfer of platelet-destroying antibodies. The newborn has small purplish marks on the skin due to bleeding beneath the skin.

Transudate effusion that is formed as the result of increased hydrostatic pressure or decreased osmotic pressure; does not indicate a true pathologic state in the anatomic region.

Trisomy condition in which one daughter cell has an extra chromosome (three copies instead of two).

Tumor suppressor gene hereditary unit whose protein products function to inhibit the growth of normal cells. Both alleles of a tumor suppressor hereditary unit must be altered to lose the function of a tumor suppressor unit of heredity.

Turbidimetric clot detection measurement of the cloudiness of a fluid caused by suspension of particles.

Turnaround time period between collecting a specimen and reporting a test result.

Type 1 VWD (classic VWD) quantitative decrease of structurally normal VWF.

Type 2 VWD qualitative disorder of VWF; has four possible subtypes: 2A, 2B, 2M, and 2N.

Type 3 VWD severe, rare quantitative deficiency of VWF.

Tyrosine kinase protein compound that regulates metabolic pathways and serves as receptor for growth factors.

Ubiquitin protein found in all eukaryotic cells that becomes covalently attached to certain residues of other proteins and tags a protein for intracellular proteolytic destruction.

UIBC *See* Unsaturated iron-binding capacity

Unfractionated heparin heterogeneous mixture of sulfated glycosaminoglycans (range 5,000–30,000 daltons) obtained from extraction of porcine intestinal mucosa or bovine lung.

Unique identification number figure assigned to a patient admitted to the hospital used to identify her or him; printed on the patient's arm band.

Universal precaution preventative guideline such as requiring health care workers to use gloves when handling any body fluid to minimize the workers' potential exposure to blood-borne pathogens.

Unsaturated iron-binding capacity (UIBC) portion of transferrin that is not complexed with iron (TIBC − serum iron = UIBC).

Untranslated region (UTRs) portion of DNA on a chromosome whose bases are not involved in protein synthesis.

uPAR *See* Urokinase-type plasminogen activator.

Urokinase-type plasminogen activator (uPA) enzyme found in urine and plasma that activates plasminogen to plasmin; is used as a thrombolytic agent in the treatment of thrombosis.

Urokinase-type plasminogen activator receptor (uPAR) endothelial cell membrane receiver that binds uPA and facilitates plasminogen activation.

Variable number tandem repeat any of several identical DNA segments lying one after the other in a sequence duplicated in a genome; can vary among different individuals.

Vascular permeability property of endothelial cells of blood vessels that selectively allows for exchange of gases, nutrients, and waste products.

Vasculitis inflammation of a blood vessel.

Vasoconstriction narrowing of the lumen of blood vessels that occurs immediately after an injury to the vessel.

Vaso-occlusive crisis acute event caused by spontaneous blockage of microvasculature by rigid sickle cells; can be triggered by infection, dehydration, decreased oxygen pressure, or slow blood flow but often occurs without a known cause.

Vertical interaction up-and-down interaction involving the skeletal lattice and proteins of the erythrocyte membrane that stabilizes the lipid bilayer membrane.

Viral load the number of copies of HIV-1 RNA that indicates a patient's status usually reported in virus particles per milliliter.

Viscosity resistance to flow; physical property that depends on the friction of component molecules in a substance as they pass one another.

Vitamin K deficiency bleeding (VKDB) symptomatic VK deficiency in newborns that results in bleeding; formerly, called *hemorrhagic disease of the newborn* (*HDN*).

Vitamin K dependent having to do with a coagulation factor that requires vitamin K to be functional; such a protein includes

the prothrombin group coagulation factors, protein C, protein S, and protein Z

Vitamin K-dependent factor *See* Prothrombin group.

Vitronectin serum or extracellular-matrix glycoprotein capable of binding heparin.

von Willebrand disease autosomal dominant hereditary bleeding disorder that lacks von Willebrand factor (VWF), which is needed for platelets to adhere to collagen. Platelet aggregation is abnormal with ristocetin as is the bleeding time. The APTT can be prolonged because of a decrease in the FVIII molecule secondary to a decrease in VWF.

von Willebrand factor (VWF) plasma component that is a molecule of multimers; needed for platelets to adhere to collagen by via platelet glycoprotein Ib. It is synthesized in megakaryocytes and endothelial cells and is noncovalently linked to FVIII in plasma.

von Willebrand factor (VWF) activity (VWF:RCo; VWF:A) test that determines the ability of VWF to function in platelet adhesion. VWF:RCo measures the ability of the patient's VWF to support agglutination of normal platelets by ristocetin in a platelet aggregometer.

von Willebrand factor (VWF) antigen immunologic property of the VWF that allows the protein to be identified and measured.

von Willebrand factor (VWF):Ag assay immunologic test that determines the quantity of VWF protein.

von Willebrand factor (VWF) multimer VWF molecule consisting of multiple copies of identical subunits linked together.

Waldenström macroglobulinemia combination of lymphoplasmacytic lymphoma (LPL) with bone marrow involvement and an IgM monoclonal paraprotein.

Warm autoimmune hemolytic anemia condition resulting from the presence of IgG autoantibodies that are reactive at 37°C with antigens on subject's erythrocytes. The antibody–antigen complex on the cell membrane sensitizes the erythrocyte, which is removed in the spleen or liver.

Wedge smear blood preparation on a glass microscope slide by placing a drop of blood at one end and using a second slide to pull the blood the length of the slide.

White blood cell (WBC) cellular component of blood with five major types: neutrophils, eosinophils, basophils, lymphocytes, and monocytes. It develops from the hematopoietic stem cell under the influence of cytokines (growth factors) and functions to defend the body against foreign antigens such as bacteria. The reference interval is $4.5–11.0 \times 10^9$/L. Also called *leukocyte.*

White thrombus blood clot formed in arteries where blood flow is rapid; is composed primarily of platelets and fibrin with relatively few leukocytes and erythrocytes.

Wiskott-Aldrich syndrome/X-linked thrombocytopenia (WAS/XLT) disorder of small platelets, thrombocytopenia, and severe immune dysregulation; involves mutations of the *WAS* gene, which manifests as isolated thrombocytopenia without immune dysfunction.

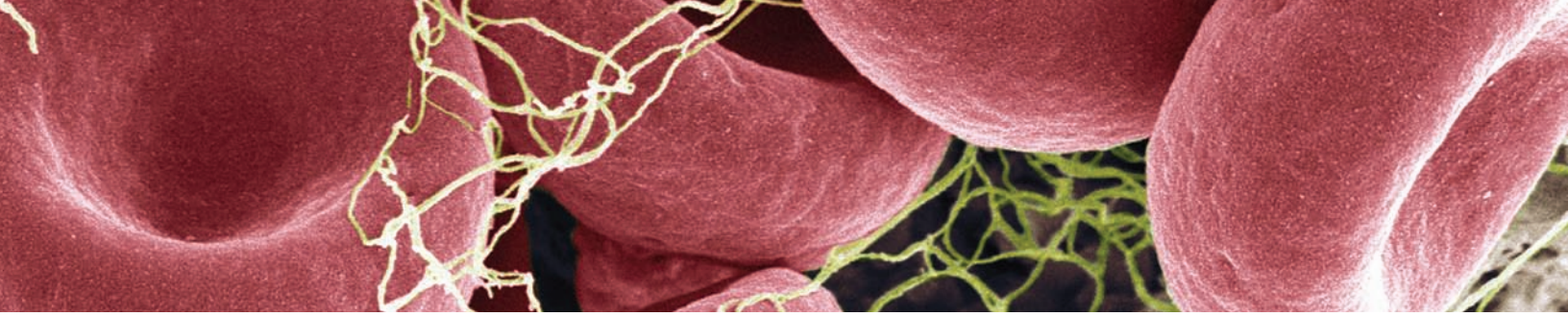
World Health Organization (WHO) 2008 classification grouping system for neoplastic blood disorders revised in 2008 that uses the test results of cytogenetics, molecular genetics and flow cytometry together with cell morphology to identify and classify hematopoietic and lymphoid neoplasms.

X-linked thrombocytopenia (XLT) disorder involving mutations of the *WAS* gene, which manifests as isolated thrombocytopenia without immune dysfunction.

y-intercept point where the regression line intersects the y-axis; is used to identify the presence of constant systematic error.

Zygosity state of being homozygous or heterozygous for a gene.

Zymogen inactive precursor that can be converted to the active form by an enzyme, alkali, or acid; its inert coagulation factors are zymogens. Also called *proenzyme.*



Index

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z

★ TABLE D Reference Values for Common Coagulation Test Reference Values[a,b]

Analyte	Reference Value
Activated partial thromboplastin time (APTT)	36–40 seconds (birth to 1 week) 25–35 seconds (1 week to adult)
Prothrombin time	11.9–15.1 seconds
	0.8–1.2 INR (6 months to adult)
	2.0–3.0 INR range for anticoagulant therapy for venous thrombosis
	2.5–3.5 INR range for anticoagulant therapy for mechanical heart valve
Bleeding Time	1–7 minutes
Thrombin time	15–19 seconds
Fibrinogen	200–400 mg/dL (2.0–4.0 g/L)
Reptilase time	18–22 seconds
Protein S functional	55–174%
Protein S free	70–140%
Protein S total	70–140%
Protein C functional	70–140%
Protein C antigen	65–150%
FII	70–145%
FV	60–150%
FVII	65–140%
FVIII	50–150%
FIX	50–160%
FX	70–150%
FXI	70–130%
FXII	50–160%
Factor II, V, VII, VIII, IX, X, XI inhibitors	<0.5 BU/mL
D-dimer	<400 ng/L
Dilute Russell's viper venom time (DRVVT)	<40 seconds
Hexagonal phase correction	<8 seconds
Lupus anticoagulant	negative
Antithrombin (AT)	80–125%
AT	
Antigen	22–40 mg/dL
Activity	80–120%
Activated protein C resistance (APCR)	>2.1
Plasminogen	76–124 (males)
	65–153% (females)
Plasminogen activator inhibitor-1 (PAI-1)	
Activity	78–142%
Antigen	4–43 mcg/mL
von Willebrand factor activity	42–139%
von Willebrand factor antigen	60–150%

[a]Compiled from multiple sources.

[b]Results vary depending on reagent and instrument combinations used in testing.

★ TABLE E Reference Values for Tests to Monitor Erythrocyte Destruction

Analyte	Age	Conventional Unit
Bilirubin	<1 day	1–6 mg/dL
	1–2 days	6–7.5 mg/dL
	2–<5 days	4–8 mg/dL
	>5 days to adult	0.2–1.0 mg/dL total (high critical limit 15 mg/dL with 5 SD)
		0–0.2 mg/dL (conjugated)
Haptoglobin	—	
Female		40–175 mg/dL (400–1750 mg/L)
Male		35–164 mg/dL (350–1640 mg/L)

★ TABLE F Reference Intervals for Iron Analytes[a]

Analyte	Age	Sex	Conventional Units
Serum iron	<1 month	—	60–200 mcg/dL
	1mo–1 year	—	15–160 mcg/dL
	1–17 years	—	50–120 mcg/dL
	Adult	Male	65–180 mcg/dL
	Adult	Female	50–180 mcg/dL
Serum ferritin	Newborn	—	25–200 mcg/L
	6 months–15 years	—	7–142 mcg/L
	>15 years	Male	20–300 mcg/L
	>15 years	Female	12–200 mcg/L
Iron deficient	>15 years	—	<35 mcg/L
Iron overload	>15 years	—	>335 mcg/L
TIBC	Adult	—	250–450 mcg/dL
UIBC	Adult	Male	132–323 mcg/dL
	Adult	Female	144–412 mcg/dL
Percent saturation	Adult	Male	20–50%
	Adult	Female	15–50%
sTfR	Adult	—	8.7–28.1 nmol/L
TfR-Ferritin index			0.63–1.8

[a]Compiled from multiple sources.

★ TABLE G Reference Values for Other Common Tests to Differentiate Causes of Anemia[a]

Analysis	Conventional Unit
Hemoglobin electrophoresis	Hb A >95%
	Hb A_2 1.5–3.7%
	Hb F 0–2.0% (>1 year)
	0–85% (birth to 1 year)
Vitamin B_{12}	150–450 pg/mL (decreases significantly with age in males)
Serum folate	>2.5 ng/mL (adults)
	>3.0 ng/mL (1-year-old children)
	>6.5 ng/mL (1–10-year-old children)
RBC folate	160–700 ng/mL (adults)
	75–1000 ng/mL (children <1 year old)
	95–365 ng/mL (children 1–11 years old)
Erythropoietin (EPO)	3–16 IU/L (SI unit)
Methemoglobin	0.06–0.24 g/dL (9.3–37.2 mmol/L)

[a]Compiled from multiple sources.

★ TABLE H Reference Values for Porphyrins and Porphyrin Precursors[a]

Analyte	Urine	Erythrocytes
Uroporphyrin	4–20 mcg/day	
Coproporphyrin	13–180 mcg/day	
Porphobilinogen	<2 mg/day	
Δ Aminolevulinic acid	1.5–7.5 mg/day	
Protoporphyrin		17–77 mcg/dL of RBCs

[a]Compiled from multiple sources.

★ TABLE I Reference Values for Normal Osmotic Fragility

Sodium Chloride (NaCl) Concentration (%)	% Hemolysis Before Incubation	% Hemolysis After Incubation
0.20	100	95–100
0.30	97–100	85–100
0.35	90–99	75–100
0.40	50–95	65–100
0.45	5–45	55–95
0.50	0–6	40–85
0.55	0	15–70
0.60	0	0–40
0.65	0	0–10
0.70	0	0–5
0.75	0	0

Increased osmotic fragility, as occurs in the presence of spherocytes, shows hemolysis beginning at 0.65–0.60% NaCl and is complete at about 0.45–0.40% before incubation. Some forms of hereditary spherocytosis will not show increased osmotic fragility until the cells are incubated at 37°C for 24 hours before running this test. After incubation these abnormal cells will begin to lyse at 0.65–0.70% NaCl, and lysis is complete at about 0.55%.

★ TABLE J Reference Values for Leukocyte Alkaline Phosphatase (LAP) Scores

Interpretation	Score
Normal	13–130
Chronic myelogenous leukemia	0–13
Polycythemia vera, infection, myeloid metaplasia	Upper range of normal or elevated
Paroxysmal nocturnal hemoglobinuria	Very low